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(54) Title: METHODS AND COMPOSITIONS FOR THE MANUFACTURE OF MOLECULAR BEACONS

(57) Abstract: Ligation-based assembly of oligonucleotides to produce molecular beacons is provided. Formation of molecular beacons is monitored to improve reaction yield and efficiency and to permit optimization of structurally similar molecular beacons. Ligation mixtures and libraries of molecular beacon components are also provided. Methods of detecting juxtaposed nucleic acids using modular molecular beacons such as intron-exon junctions are provided.

METHODS AND COMPOSITIONS FOR THE MANUFACTURE OF MOLECULAR BEACONS

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CROSS REFERENCE TO RELATED APPLICATIONS

This application is a non-provisional of Application USSN 60/200,333

filed April 28, 2000. *The present application claims priority to and benefit of this prior*

application, pursuant to 35 U.S.C. 119, as well as any other applicable statute or rule.

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FIELD OF THE INVENTION

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The present invention is in the field of molecular beacon synthesis and use

for detection of target sequences, including juxtaposed sequences produced by splicing or

cloning.

BACKGROUND OF THE INVENTION

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Molecular beacons (MBs) are oligonucleotides designed for the detection

and quantification of target nucleic acids (e.g., target DNAs). The basic principles of

molecular beacon mediated target nucleic acid detection is depicted in **Figure 1.**

As depicted, 5' and 3' termini of the MB collectively comprise a pair of

moieties which confers detectable properties of the MB. As shown, one of the termini is

25   attached to a fluorophore and the other is attached to a quencher molecule capable of

quenching a fluorescent emission of the fluorophore. For example, one example

fluorophore-quencher pair can use a fluorophore such as EDANS or fluorescein, e.g., on

the 5'-end and a quencher such as Dabcyl, e.g., on the 3'-end.

When the MB is present free in solution, i.e., not hybridized to a second

30   nucleic acid, the stem of the MB is stabilized by complementary base pairing. This self-

complementary pairing results in a "hairpin loop" structure for the MB in which the

fluorophore and the quenching moieties are proximal to one another. In this

confirmation, the fluorescent moiety is quenched by the fluorophore.

5   The loop of the molecular beacon is complementary to a sequence to be detected in the target nucleic acid, such that hybridization of the loop to its complementary sequence in the target forces disassociation of the stem, thereby distancing the fluorophore and quencher from each other. This results in unquenching of the fluorophore, causing an increase in fluorescence of the MB.

10   Further details regarding standard methods of making and using MBs are well established in the literature and MBs are available from a number of commercial reagent sources. Further details regarding methods of MB manufacture and use are found, e.g., in Leone et al. (1995) "Molecular beacon probes combined with amplification by NASBA enable homogenous real-time detection of RNA." Nucleic Acids Res.

15 26:2150-2155; Tyagi and Kramer (1996) "Molecular beacons: probes that fluoresce upon hybridization" Nature Biotechnology 14:303-308; Blok and Kramer (1997) "Amplifiable hybridization probes containing a molecular switch" Mol Cell Probes 11:187-194; Hsuih et al. (1997) "Novel, ligation-dependent PCR assay for detection of hepatitis C in serum" J Clin Microbiol 34:501-507; Kostrikis et al. (1998) "Molecular beacons: spectral

20 genotyping of human alleles" Science 279:1228-1229; Sokol et al. (1998) "Real time detection of DNA:RNA hybridization in living cells" Proc. Natl. Acad. Sci. U.S.A. 95:11538-11543; Tyagi et al. (1998) "Multicolor molecular beacons for allele discrimination" Nature Biotechnology 16:49-53; Bonnet et al. (1999) "Thermodynamic basis of the chemical specificity of structured DNA probes" Proc. Natl. Acad. Sci. U.S.A.

25 96:6171-6176; Fang et al. (1999) "Designing a novel molecular beacon for surface-immobilized DNA hybridization studies" J. Am. Chem. Soc. 121:2921-2922; Marras et al. (1999) "Multiplex detection of single-nucleotide variation using molecular beacons" Genet. Anal. Biomol. Eng. 14:151-156; and Vet et al. (1999) "Multiplex detection of four pathogenic retroviruses using molecular beacons" Proc. Natl. Acad. Sci. U.S.A. 96:6394-

30 6399. Additional details regarding MB construction and use is found in the patent literature, e.g., USP 5,925,517 (July 20, 1999) to Tyagi et al. entitled "Detectably labeled dual conformation oligonucleotide probes, assays and kits;" USP 6,150,097 to Tyagi et al (November 21, 2000) entitled "Nucleic acid detection probes having non-FRET fluorescence quenching and kits and assays including such probes" and USP 6,037,130 to

35 Tyagi et al (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in assays and kits."

5   MBs are gaining wide spread acceptance as robust reagents for detecting
and quantitating nucleic acids, including in real time (e.g., MBs can be used to detect
targets as they are formed). A variety of commercial suppliers produce standard and
custom molecular beacons, including Chruachem (chruachem.com), Oswel Research
Products Ltd. (UK; oswel.com), Research Genetics (a division of Invitrogen, Huntsville

10   AL (resgen.com)), the Midland Certified Reagent Company (Midland, TX mcrc.com) and
Gorilla Genomics, LLC (Alameda, CA). A variety of kits which utilize molecular
beacons are also commercially available, such as the Sentinel™ Molecular Beacon
Allelic Discrimination Kits from Stratagene (La Jolla, CA) and various kits from
Eurogentec SA (Belgium, eurogentec.com) and Isogen Bioscience BV (The Netherlands,

15   isogen.com).
          Despite such widespread acceptance and commercial development of MBs
and related technologies, there remain a number of areas for improvement in the design,
manufacture, synthesis, and purification of MBs. For example, in the area of single
nucleotide polymorphism (SNP) detection, one typically designs, tests and synthesizes

20   MBs separately for each SNP. This is, of course, inefficient and expensive at several
levels. For example, the design and testing process is labor intensive. Additionally, it is
difficult to scale the amount of MB actually needed to the synthesis scheme used to make
the MB. That is, it can be difficult to scale a synthetic reaction down to produce only as
much material as is actually needed—which, with the evolution of modern laboratory

25   systems that run and detect reactions in nanoliter volumes, can be extremely small indeed.
          Further in this regard, there are a number of specific difficulties with
current synthetic schemes for making MBs. First, MB oligonucleotides require labels on
both the 5' and 3' ends of the oligonucleotide. Adding 5' and 3' labels to
oligonucleotides increases their cost dramatically, since specialized CPG (controlled pore

30   glass) supports for solid-phase synthesis are typically used for the 3' attachment, and
specialized phosphoramidites are required for the 5' attachment. Second, MBs are
generally long oligonucleotides (typically greater than 30 nucleotides in length). The
longer an oligonucleotide, the lower the percentage of final oligonucleotide product
which is full-length, due to the compounding likelihood of synthesis failure at each base.

35   Oligonucleotide purity, therefore, decreases as a function of oligonucleotide length,
reducing the effectiveness of the MB and increasing the requirement for purification
following synthesis. Indeed, typically, MB oligonucleotides are purified to operate

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5   according to specifications. Purification of oligonucleotides that differ by one or a few

       bases in length is best achieved by polyacrylamide gel electrophoresis (PAGE)-based

       methods, which are relatively labor intensive and, therefore, expensive. Finally, once

       designed and synthesized, there is a significant probability that a given MB will be

       ineffective, due to interfering secondary structure in its own loop region, or interfering

10   secondary structure in the sequence of the target DNA to which the MB hybridizes, which

       interferes with the hybridization of the MB and target sequence.

              The present invention uses modular synthesis strategies to overcome

       scalability, purification and synthesis issues noted above and to substantially decrease the

       amount of time needed to design and test MBs. Libraries; kits, devices, ligation mixtures

15   and methods to achieve these goals are provided.

              A fuller understanding of the invention will be provided by review of the

       following.

SUMMARY OF THE INVENTION

              The present invention uses ligation-based assembly to make MBs. That is,

20   MB components such as the stem, loop, label and label quenching moieties are made

       separately and then assembled by chemical or enzymatic ligation. Basic approaches

       include template-based, multiple template based and non-template based ligation

       assembly reactions. The MBs and components used to make the MBs can comprise

       nucleic acids, peptide nucleic acids, or both. Most typically, ligation dependent changes

25   in label output are used to monitor ligation of MB components. Methods, devices,

       ligation mixtures and libraries are provided for high-throughput synthesis and ligation

       optimization.

              Accordingly, the invention comprises methods of making one or more

       molecular beacon or molecular beacon component (a sub part of a complete molecular

30   beacon). In the methods, a first oligonucleotide or peptide nucleic acid (PNA)

       corresponding to a first subsequence of a molecular beacon is provided (e.g., by

       synthesizing the component). At least a second oligonucleotide or PNA corresponding to

       a second subsequence of a molecular beacon is also provided (as set forth in more detail

       below, the MB can be made by ligation of 2 or more elements). The first and second

35   oligonucleotides or PNAs are ligated together, thereby forming the molecular beacon (or

       the molecular beacon component, where the ligation scheme uses more than 2

5   oligonucleotides to make the MB). One or more additional oligonucleotide or PNA can
       also be included in the ligation reaction to produce the MB or MB component.

               Most typically, the first oligonucleotide or PNA includes a label moiety
       and the second oligonucleotide includes a label quenching moiety. Common label
       moieties include those derived from Texas red, terbium chelate, europium cryptate,
10   Fluorescein, IAEDANS, EDANS, BODIPY FL or the like. Common quenching moieties
       include TRITC (tetrarhodamine isothiocyanate), Allophycocyanin, EDANS,
       Tetramethylrhodamine, DABCYL, Fluorescein, BODIPY FL, QSY 7 dye or the like.

               In a significant aspect, the method optionally includes monitoring a
       ligation-dependent change in a signal output of the molecular beacon, or of the first or
15   second oligonucleotide or PNA. The ligation-dependent signal output is, e.g., a change in
       a fluorescence emission at a hybridization temperature that permits intra-molecular
       hybridization of the molecular beacon, but does not permit inter-molecular hybridization
       of the molecular beacon. The fluorescence emission change correlates to synthesis of the
       MB. Detection of this change can be used for a variety of purposes including optimizing
20   one or more reaction parameters to increase yield of the molecular beacon or to improve
       the efficiency of the ligating step. Similarly, one or more reaction parameters can be
       optimized to minimize an amount of unligated material remaining following the ligating
       step.

               Detection of the ligation-dependent change (i.e., formation of the MB from
25   separate oligonucleotides) is dependent on the melting and self-annealing of any MB that
       is actually assembled. Thus, the invention optionally includes using melting and
       annealing profiles of ligation-dependent emission changes to identify one or more MBs
       that have an optimized structural component (loop or stem) sequence.

               As noted, both template and non-template dependent ligation reactions can
30   be used. For example, the first and second oligonucleotides or PNAs can be aligned on a
       template nucleic acid prior to said ligating step. The template oligonucleotide can
       participate in the ligation reaction (thus becoming part of the final MB) or can not
       participate in the reaction. In this later embodiment, the ends of the oligonucleotide or
       PNA can be structured to prevent ligation, e.g., in the case of an oligonucleotide by not
35   including phosphate or hydroxyl groups at the terminus of the oligonucleotide. Most
       commonly, the template nucleic acid is a synthetic single-stranded oligonucleotide,
       though, e.g., PNAs or cloned nucleic acids can also be used to align MB components in

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5   the ligation reaction. Typically, the ligating step is performed via enzymatic ligation,
    though chemical ligation approaches can also be used. Common ligase enzymes suitable
    for the ligation reaction include Taq DNA ligase, E. coli DNA ligase, and T4 DNA ligase.

            One advantage of the present invention is that purification of the MB from
    components used to make the MB is simplified due to the substantial difference in size
10 between the MB and the oligos or PNAs used to make the MB. Thus, one aspect of the
    invention includes purifying the molecular beacon from one or more unligated first or
    second oligonucleotides or PNAs. Common purification methods include simple
    purification methods such as HPLC, ion-exchange chromatography or the like.

            It will be appreciated from the foregoing that ligation mixtures, e.g.,
15 contained in device comprising detectors for monitoring ligation-dependent changes in
    MB signal output, as well as libraries of ligation components, e.g., used in the methods
    are also a feature of the invention. For example, ligation mixtures that include a first
    oligonucleotide or PNA comprising a label moiety, a second oligonucleotide or PNA
    comprising a quenching moiety that quenches the label moiety when placed proximal or
20 in contact with the label moiety, a third oligonucleotide or PNA that is at least partly
    complementary to at least a portion of the first or second oligonucleotides, and a ligase
    are a feature of the invention. The first and second oligonucleotides or PNAs can also be
    at least partly complementary. Ligation of the first, second and third oligonucleotides or
    PNAs can result in formation of a molecular beacon, or the third oligo or PNA can simply
25 be a template used in the ligation reaction (of course, additional oligos can be used as MB
    component elements, or as additional ligation templates). Thus, in one embodiment,
    ligation of the first and second oligonucleotides or PNAs results in formation of a
    molecular beacon with the third oligonucleotide providing a template for ligation of the
    first and second oligonucleotides. In another embodiment, nucleotides of the first and
30 second oligonucleotides form at least a portion of a molecular beacon stem and
    nucleotides of the third oligonucleotide forms at least portion of a hairpin loop portion of
    the molecular beacon. These ligation mixtures can be formed in a device having a
    ligation reaction region, e.g., a micotiter tray, test-tube, cuvette, microfluidic component
    or other structure configured to receive the ligation reaction.

35   In the embodiments above, the second oligonucleotide or PNA is typically
    at least partly complementary to one or more target nucleic acid, e.g., at least partly
    complementary to one or more single nucleotide polymorphism (SNP). Thus, the MBs

5   made according to the present invention can be used to detect a target nucleic acid such as
       a SNP, RNA, DNA or the like.

               As noted above, a variety of label and quencher elements can be
       incorporated into the ligation mixture on either the PNA or oligonucleotide, including
       labels such as Texas red, terbium chelate, europium cryptate, Fluorescein, IAEDANS,

10   EDANS, and BODIPY FL and quenchers such as TRITC (tetrarhodamine
       isothiocyanate), Allophycocyanin, EDANS, Tetramethylrhodamine, DABCYL,
       Fluorescein, BODIPY FL, and QSY 7 dyes. Again, ligases that can be used include E.
       coli ligase, T4 ligase, Taq ligase and other known ligases. Ligation buffers, e.g., selected
       to facilitate operation of the ligase enzymes can be included as part of the mixture.

15   Kits comprising ligation mixture components (typically in unmixed form)
       and kit components (packaging materials, instructions for using the components to
       produce one or more molecular beacons, or one or more containers (microtiter trays,
       eppendorf tubes, etc.) for holding the components are also a feature of the invention.
       Standards for calibrating any MB detection reaction such as standard target sequences,

20   amplification primers for amplifying a target sequence, or the like, can also be included in
       the kits of the invention.

               One additional feature of the invention includes libraries of molecular
       beacon components. The libraries are designed, e.g., for the rapid synthesis of variants of
       an MB, e.g., to test variants against one or more target sequences. Such libraries include,

25   e.g., a set of a plurality of hairpin loop oligonucleotides or PNAs, each of the plurality of
       hairpin loop oligonucleotides or PNAs comprising a subsequence of at least one
       molecular beacon, the subsequence comprising less than all of the molecular beacon, and
       at least one label or label quenching oligonucleotide or PNA. The oligonucleotide or
       PNA comprises at least one label or label quenching moiety, where ligation of at least one

30   hairpin oligonucleotide or PNA and the label or label quenching oligonucleotide or PNA
       produces a molecular beacon or molecular beacon subsequence.

               Most typically, the library is formatted in a gridded array, such as a
       microtiter tray, to facilitate access to the components of the library. However, any
       logically accessible arrangement can be used for the library. Thus, for example,

35   individual members types of the hairpin loop oligonucleotides or PNAs are located in
       wells of the microtiter tray, with the other MB components being added to the wells for
       ligation to form MBs.

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5   In one aspect, the hairpin loop oligonucleotide or PNA has a label or label
quenching moiety, and ligation of the hairpin loop oligonucleotide or PNA to the label or
label quenching oligonucleotide or PNA produces a molecular beacon. In another aspect,
the library includes both a label oligonucleotide or PNA and a label quenching
oligonucleotide or PNA, where ligation of the label oligonucleotide or PNA, the label
10   quenching oligonucleotide or PNA and the hairpin loop oligonucleotide or PNA produces
a molecular beacon.

The library can also include essentially any component of the ligation
reaction described above, including enzymes, buffers, and the like. The libraries can also
be made and used in kit form, e.g., providing the library in conjunction with packaging
15   materials, instructions for using the library to produce one or more molecular beacons,
one or more containers for holding one or more components of the library, one or more
ligase enzyme, one or more standard target molecule, one or more amplification
oligonucleotides, one or more ligation buffer, or the like.

In one embodiment, the invention provides methods for detecting
20   juxtaposition of two or more target subsequences in a target nucleic acid, e.g., as occurs
in RNA splicing (or RNA splicing and reverse transcription, as in cDNA production),
cloning or the like. In this class of embodiments, a molecular beacon is formed by
ligating a first oligonucleotide complementary to a first target subsequence and a second
oligonucleotide complementary to a second target subsequence of the target nucleic acid.
25   The resulting molecular beacon is hybridized to the target nucleic acid and a target-
specific hybridization of the molecular beacon to the first and second subsequences is
detected.

As noted above, a number of modular MB synthesis strategies are set forth
herein. These typically include ligating the oligos that form the MB by aligning the
30   oligonucleotides on one or more template and incubating the resulting hybridized set of
oligonucleotides with a ligase. A MB used for juxtaposition detection can be formed
from more than one oligo, i.e., one or more additional oligonucleotides can be ligated to
the first and/or second oligonucleotides. For example, standard stem oligos comprising
labels or label quenching moieties can be ligated.

35   **BRIEF DESCRIPTION OF THE FIGURES**
Figure 1 is a schematic of a general scheme depicting hybridization of a
MB to a target.

Figure 2 is a schematic of a sequence of Oligo A.

Figure 3 is a schematic of a sequence of Oligo B.

Figure 4 is a schematic showing complementarity of Oligos A and B comprising a partial stem component of the example MB.

Figure 5 is a schematic of a sequence of Oligo C showing the partial stem and loop structure of a MB component of the example MB.

Figure 6 is a schematic of an annealing of Oligos A, B and C.

Figure 7 is a schematic of a ligation of Oligos A, B and C.

Figure 8 is a schematic of a sequence of Oligo D.

Figure 9 is a schematic of a sequence of Oligo E.

Figure 10 is a schematic of a sequence of Oligo F.

Figure 11 is a schematic showing complementarity of Oligos D and F and Oligos E and F comprising partial components of the example MB.

Figure 12 is a schematic showing hybridization of Oligos D-F.

Figure 13 is a schematic of the conversion of the hybridized three oligo structure in Figure 12 into a MB.

Figure 14 is a schematic of a more detailed view of a MB.

Figure 15, panels A-B provide a schematic of a detection method for monitoring MB formation in a template-independent ligation reaction.

Figure 16, panels A-C provide a schematic detailing a detection method for monitoring MB formation in a template-independent ligation reaction.

Figure 17 is a schematic of a sequence of Oligo D.

Figure 18 is a schematic of a sequence of Oligo H, and includes sequence complementary to a target (underline), and a template ligation sequence (double underline).

Figure 19 is a schematic of a sequence of Oligo G, and includes sequence complementary to a target (underline), and a template ligation sequence (double underline).

Figure 20 is a schematic of Oligo G annealing to a sequence in exon 1 that is 5' to a splice junction.

Figure 21 is a schematic of Oligo H annealing to a sequence in exon 3 that is 3' to a splice junction.

Figure 22 is a schematic of a sequence of Oligo F.

9

5   Figure 23 is a schematic showing hybridization of Oligos D, G and F. comprising partial components and a ligation template for the assembly of the example MB.

Figure 24 is a schematic of a sequence of Oligo I.

Figure 25 is a schematic showing hybridization of Oligos G, H and I
10   comprising partial components and a ligation template for the assembly of the example MB.

Figure 26 is a schematic showing hybridization of Oligos D, F, G, H and I comprising partial components and a ligation templates for the assembly of the example MB.

15   Figure 27 is a schematic of the conversion of the hybridized five oligo structure in Figure 26 into the example MB.

Figure 28 is a schematic of two alternative splicing reactions.

Figure 29 is a schematic of a general scheme depicting hybridization of the example MB to exon junction target sequences.

20   Figure 30 is a schematic of a detection apparatus for detecting signals, e.g., from one or more ligation mixtures.

DEFINITIONS

The following definitions supplement those in the art.

An "oligonucleotide" is a polymer of nucleotides. The polymer can
25   additionally comprise non-nucleotide elements such as labels, quenchers, blocking groups, or the like. The nucleotides of the oligonucleotide can be natural or non-natural and can be substituted or modified.

A peptide nucleic acid (PNA) is a polymer of peptide nucleic acid monomers. The polymer can additionally comprise elements such as labels, quenchers,
30   blocking groups, or the like. The monomers of the PNA can be substituted or modified. The term "nucleic acid" encompasses any physical string of monomer units that can be corresponded to a string of nucleotides, including a polymer of nucleotides, PNAs, modified oligonucleotides and the like.

A molecular beacon (MB) is an oligonucleotide or PNA which, under
35   appropriate hybridization conditions, self-hybridizes to form a stem and loop structure. The MB has a label and a quencher at the termini of the oligonucleotide or PNA; thus,

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5   under conditions that permit intra-molecular hybridization, the label is typically quenched (or otherwise altered) by the quencher. Under conditions where the MB does not display intra-molecular hybridization (e.g., when bound to a target nucleic acid), the MB label is unquenched.

A "stem oligonucleotide" in the context of a MB is an oligonucleotide that

10   includes nucleotides of an MB that are found in the MB stem. A "loop" or "hairpin loop" oligonucleotide is an oligonucleotide that includes nucleotides of an MB that are found in the loop portion of the MB. Similarly, a stem PNA includes monomers of a PNA MB that are found in the MB stem. A "loop" or "hairpin loop" PNA is a PNA that includes monomers of a PNA MB that are found in the loop portion of the MB.

15   A label is a moiety that facilitates detection of a molecule. Common labels in the context of the present invention include fluorescent and colorimetric labels.

A quencher is a moiety that alters a property of the label when it is in proximity to the label. Both FRET and non-FRET based quenchers are appropriate to the present invention. The quencher can actually quench an emission, but it does not have to,

20   i.e., it can simply alter some detectable property of the label, or, when proximal to the label, cause a different detectable property than when not proximal to the label.

A "library" is at least one set of physical components (e.g., MBs, MB component oligos, etc.), having two or more elements. The library can include additional components (ligation mixtures, etc.) as well.

25   **DETAILED DISCUSSION**
The invention described herein provides a simplified approach to making MBs. In particular, this disclosure describes methods, compositions, devices and libraries for rapidly and inexpensively generating molecular beacons in a high-throughput fashion and for generating and testing sets of MBs against one or more target of interest.

30   In particular, methods of assembling MBs from shorter oligonucleotides are described. The methods involve ligating the shorter oligonucleotides to form the components of the MB, i.e., sequences which form the stem-loop structure and which have appropriate quencher and fluorophore components. Two example approaches are described in some detail below, i.e., a template-independent ligation approach and a

35   template mediated ligation approach.
In the template-independent ligation approach, the MB is formed by ligation of 3 (or more) component oligonucleotides: a first oligo that comprises a

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5   fluorophore and a sequence which forms a portion of the stem structure; a second

oligonucleotide that comprises a quenching moiety; a portion of the stem structure and a

third sequence which forms a portion of the stem structure and a portion of the hairpin

structure. This approach allows batch production of the first and second oligonucleotides,

with only the third oligonucleotide (which includes the portion of the MB which is

10   specific for a target of interest) being custom synthesized (of course, this target

complementary sequence can also be made from multiple oligos, and examples of such

approaches are provided below). In addition, because the unligated components are much

smaller than the fully ligated MB, purification of the final product MB is considerably

simplified. Indeed, less purification of the components is also needed, because the

15   ligation reaction itself selects against ligation of incomplete component oligonucleotides.

In the template-mediated assembly approach, the MB is formed by ligation

of two (or optionally more) oligos, which are aligned on one or more standard template

oligonucleotide(s) to place the 3' and 5' ends of the two oligos into proximity for the

ligation reaction to occur. The first oligonucleotide (and/ or additional oligonucleotides,

20   if additional oligos are involved in the ligation to be performed) and the template

oligonucleotide(s) can be batch synthesized, with only the second oligonucleotide (which

includes the portion of the MB which is specific for a target of interest) being custom

made (as is noted in more detail below, target regions can be broken into more than one

oligo; thus, more than one oligo can be custom synthesized, depending on the exact

25   format at issue). Here again, the alignment/ ligation portion of the synthesis helps prevent

incomplete MB synthesis, reducing the need for purification of the component elements

of the MB. Furthermore, as with the preceding approach, final purification of the MB

from its component elements is simplified by the substantial size difference between the

MB and the oligos used to synthesize the MB.

30   With either of these approaches it is, of course, possible to further logically

fragment the MB, i.e., to use more than 2 or 3 oligos in the synthesis of the MB. For

example, one could align 3, 4, or even more oligos on one or more template oligo(s),

allowing ligase to join multiple oligos on the template(s). However, such additional

oligos are not necessary. Similarly, a template-independent ligation approach can be

35   combined with the template-mediated ligation approach to create MBs or component

elements of the MBs, though, again, this is not necessary.

5   The following section describes the basic approaches in more detail. One

of skill will recognize a variety of features and components that can be substituted to

achieve similar results.

LIGATION-BASED MB SYNTHESIS

Template-Independent Ligation

10   One basic class of embodiments is illustrated in **Figures 2-7**, involving the

steps described therein and below. The sequences shown in the Figures are meant to be

for illustration of the principle only, not to limit the application to the illustrated concepts.

Any sequences which satisfy basic complementarity principles can be substituted.

           As illustrated, a first short oligonucleotide is synthesized with a

15   fluorophore (or quencher) molecule attached to its 5' end, by conventional methods of

DNA synthesis as known by those skilled in the art ("Oligo A," **Figure 2**).

           A second oligonucleotide is synthesized with a quencher (or fluorophore)

attached to its 3' end, and also bearing a 5' phosphate group, also by conventional

methods of DNA synthesis as known by those skilled in the art ("Oligo B," **Figure 3**).

20   Oligonucleotides A and B are designed such that they are self-

complementary and form a partial stem of a MB, as illustrated in **Figure 4**, in such a way

that the fluorophore and quencher molecule are situated at the blunt end of the double-

stranded stem, to minimize the distance between the two molecules and thereby maximize

the quenching of the fluorophore by the quencher. Furthermore, oligonucleotides A and

25   B are designed such that one of the two oligonucleotides has an overhang great enough to

permit hybridization of a third oligonucleotide, oligonucleotide C, as described below.

           A third oligonucleotide is synthesized which comprises the loop and part

of the stem of the MB, which bears a 5' phosphate group, by conventional methods of

DNA synthesis ("Oligo C," **Figure 5**). Oligo C is also designed to have an overhang

30   which is complementary to the overhang of the Oligo A: Oligo B duplex, such that the

three oligonucleotides together form the hybridized structure illustrated in **Figure 6**, in

which a junction between the two double-stranded regions positions the 5' phosphate

group of Oligo B immediately adjacent to the 3' hydroxyl group of Oligo C, and positions

the 5' phosphate group of Oligo C immediately adjacent to the 3' hydroxyl group of

35   Oligo A. In this orientation, the three oligonucleotides can be ligated into a single

oligonucleotide MB. A variation of the synthesis of Oligo C which does not require the

5   addition of a 5' phosphate group to the oligonucleotide is to make a longer sequence
     which contains a self-complementary stem structure with a site for a restriction enzyme,
     which is then digested to generate the desired complementary overhang.

             Enzymatic ligation, using an enzyme such as T4 ligase, *E. coli* ligase, a
     thermostable ligase, or any other enzyme capable of ligating nicks in double-stranded
10   DNA molecule, is then used to convert the hybridized structure composed of Oligo A,
     Oligo B, and Oligo C into a single oligonucleotide (Oligo A-C-B), as shown in **Figure 7.**
     Temperatures and salt concentrations of the ligation reaction are maintained such that the
     complementary overhangs are kept in close proximity. Ligation conditions can be
     manipulated in various well-known ways for maximizing the efficiency of the reaction.

15   Lastly, the ligated structure Oligo A-C-B is purified away from the
     individual components, which is easily achieved due to its greater length. Purification can
     be achieved either by reverse-phase HPLC, or by many other means, including gel
     filtration, ion exchange chromatography, or by other means known to those skilled in the
     art.

20   Advantages of this class of embodiments include the following.

             First, Oligo A and Oligo B, possessing fluorophore and quencher
     molecules, need only be made once, as they can be used in the manufacture of numerous
     different MBs, which will differ only in the sequence of Oligo C. As long as Oligo C
     always possesses an overhang which is complementary to the overhang of Oligo A: Oligo
25   B, the expense of synthetically adding 3' and 5' labels to each MB can be avoided.
     Second, purification of MBs is simplified by the fact that the final product A-C-B is
     considerably longer than A, B, or C alone. In other words, in any chromatographic
     purification, the separation of the desired product will be straightforward and PAGE-
     based separation is not necessary to produce highly pure MBs. Oligos A and B need only
30   be purified once, and they can be made in a relatively large batch (which simplifies
     purification). Moreover, many of the n-1 and other incomplete versions of Oligo C do
     not have an appropriate overhang for hybridization and ligation, and, therefore, are not
     incorporated into the MB. Third, the simplicity and low cost of the method provides for
     the affordable generation of MBs in a high-throughput (e.g., 96 or 384 (or greater
35   number) well microtiter tray, or even microfluidic) format. Fourth, high-throughput
     assembly of MBs allows multiple MBs to be generated and tested as hybridization probes
     for a given target sequence (e.g., for SNP discrimination).

                                             14

5   <u>Template-Mediated Ligation</u>
         In a second class of embodiments which utilizes a template for MB

assembly, a first short oligonucleotide is synthesized with a fluorophore molecule

attached to its 5' end, again, by conventional methods of DNA synthesis ("Oligo D", see

Figure 8). Again, an exact sequence shown is for illustrative purposes, but could be

10   entirely different. Oligo D will, however, be complementary to Oligo F as shown in

Figure 11. In **Figure 8**, the fluorophore is indicated as "F." In the final product MB, the

oligo sequence comprises all or part of one half of the beacon's "stem" sequence. The

sequences shown here, which are complementary to Oligo F, are 6 ucleotides (nt) in

length, but could be as short as about 4 nt or as long as about 10 nt, and still effectively

15   anneal to a complementary Oligo F.

         A second oligonucleotide is synthesized with a quencher attached to its 3'

end, and also bearing a 5' phosphate group, by conventional methods of DNA synthesis

as known by those skilled in the art ("Oligo E", **Figure 9**). This second oligo also

includes a target-specific sequence which becomes the "loop" of the molecular beacon.

20   Again, the exact sequence shown is for illustrative purposes, and could be different, but is

complementary to Oligo F as shown in **Figure 11**. The quencher is indicated as "Q."

The region indicated by the line in the middle of the sequence indicates the variable loop

region of the beacon, which is typically in the range of 17 to 25 nucleotides in length, but

can be shorter or longer. The region 3' to the variable loop region comprises all of part of

25   one half of the MB "stem" sequence, and is complementary to Oligo D. The region 5' to

the variable loop region is complementary to Oligo F. The sequence shown in this

example is 5 nt in length, but can be as short as 4 nt or as long as 10 nt, and still

effectively anneal to a complementary Oligo F.

         Oligonucleotides D and E are designed such that they form a self-

30   complementary hybrid at their 5' (Oligo D) and 3' (Oligo E) ends, which form the stem

of the MB, as illustrated in **Figures 13** and **14**, in such a way that the fluorophore and

quencher molecule are situated at the blunt end of the double-stranded stem, to minimize

the distance between the two molecules and thereby maximize the quenching of the

fluorophore by the quencher. Furthermore, oligonucleotides D and E are designed such

35   that they are both complementary to a third "ligation template oligo (Oligo F).

         A third oligonucleotide (the ligation template oligonucleotide) is

synthesized which bears a blocked 3' end, by conventional methods of DNA synthesis

5   ("Oligo F", **Figure 10**). Again, an exact sequence shown is for illustrative purposes, but
     could be different, as long as it is appropriately complementary to Oligos D and E as
     shown in **Figure 11**. The ligation template oligo is blocked at its 3' end so that it can not
     take part in ligation reactions. Such 3' blockages include (for example) 3' phosphate
     group addition, or the use of a dideoxynucleotide as the 3' nucleotide in the sequence.

10   Oligo F is designed to form a duplex with both Oligo D and Oligo E, such
     that the three oligonucleotides together form the hybridized structure illustrated in
     Figures 11-12. As illustrated, the oligonucleotides form a duplex, with Oligo F acting as
     a template for ligation of Oligos D and E. The hybridization is performed at an
     appropriate stringency to provide for specific hybridization of the oligos. The

15   hybridization can be performed with a large excess of Oligo F to provide for the
     trimolecular hybrid which competes favorably against any bimolecular hybridization
     between any complementary regions of Oligos D and E.

                As shown, upon hybridization, a junction forms between two double-
     stranded regions, which positions the 5' phosphate group of Oligo E immediately

20   adjacent to the 3' hydroxyl group of Oligo D. In this orientation, Oligo D and Oligo E
     can be ligated into a single oligonucleotide MB.

                As noted above, the 3' blockage of Oligo F can be achieved by various
     methods, such as the addition of a 3' phosphate group or the use of a 3'
     dideoxynucleotide. The functional use of such a blockage is simply that it prevents Oligo

25   F from taking part in any ligation reactions. Oligo F also can be synthesized not to carry
     a 5' phosphate group, and, therefore, not to take part in ligation reactions with its 5' end.

                Enzymatic ligation, using an enzyme such as T4 ligase, *E. coli* ligase, a
     thermostable ligase, or any other enzyme capable of ligating nicks in a double-stranded
     DNA molecule, is used to convert the hybridized structure composed of Oligo D, Oligo

30   E, and Oligo F into two oligonucleotides (Oligo D-E and the "ligation template oligo"
     Oligo F), as shown in **Figure 13** and **Figure 14**. Temperatures and salt concentrations of
     the ligation reaction are maintained such that the complementary overhangs are kept in
     close proximity. Conditions of ligation can be manipulated in various ways known to
     those skilled in the art for maximizing the efficiency of the reaction.

35   As depicted, **Figure 14** provides a detailed view of the MB shown in
     Figure 13. The dashed lines from previous figures is here shown as the letter X, which
     represents any of the bases G, C, A, or T. The loop of the molecular beacons generated

5   by this method therefore include both the target-specific region (illustrated by the X's) as

well as a region which may not be target-specific (in this example, the sequence CGACG,

having been added for the purposes of effecting ligation of Oligo D to Oligo E.

Lastly, the ligated structure Oligo D-E is purified away from any

individual unligated components, and Oligo F, which is easily achieved due to its

10   substantially greater length. Purification can be achieved, e.g., by reverse-phase HPLC,

or by many other method, including gel filtration, ion exchange chromatography, or by

any other available method.

Advantages of this embodiment include the following.

First, Oligo D (which includes a fluorophore) can be made once and used

15   in the manufacture of numerous different MBs, which differ only in the sequence of the

variable loop region of Oligo E (the part of the MB which provides target specificity).

The expense of adding 5' labels to each MB can, thus, be avoided.

Second, purification of product MBs is simplified by the fact that the final

product oligo D-E is considerably longer than D, E, or F alone. In other words, in any

20   chromatographic or other simple purification, the separation of the desired product is

straightforward. Oligo D need only be purified once, since it can be made in a large

batch. Moreover, many of the "failure" sequences ("n-1" and shorter sequences) of Oligo

E do not have an appropriate 5' phosphate group and complementary region for ligation

to Oligo D and, therefore, are eliminated from any product MB. Third, the simplicity and

25   low cost of the method provides for the affordable generation of MBs in a high-

throughput (e.g., microtiter or microfluidic) format. The benefit of such a method is that

by generating multiple MBs for each sequence to be analyzed, it is possible to determine

which MBs work as effective hybridization probes, facilitating the use of MBs.

MONITORING MB SYNTHESIS IN A TEMPLATE-INDEPENDENT LIGATION
30   REACTION

The reagents and reactions illustrated in **Figures 2-7** show a method for

the modular assembly of MBs by enzymatic ligation as discussed in detail above. The

yield from ligation-based MB synthesis is directly related to the efficiency of the ligation

reaction. As the efficiency of the reaction increases, the overall yield also increases.

35   Monitoring the efficiency of each MB synthesis reaction can be done to optimize

conditions of enzymatic ligation synthesis of MB. The following discussion provides

methods for directly monitoring ligation-based MB syntheses.

5   Illustrated in **Figure 15, Panel A** are three annealed oligonucleotides labeled Oligo A, Oligo B, and Oligo C. A full-length molecular beacon is synthesized by the enzymatic ligation of the annealed component oligonucleotides, as shown in **Figure 15, Panel B**. As the efficiency of the ligation reaction increases, the percentage of the ligated product shown in **Figure 15, Panel B** also increases, and the percentage of

10   unligated component molecules shown in **Figure 15, Panel C** decreases. The annealing is performed at an optimal stringency to provide for specific self-annealing of the MB.

Melting and annealing of enzymatic ligation reactions using optimized conditions yield the ligated MB that is quenched, as shown in **Figure 15, Panel B**. Melting and annealing of enzymatic ligation reactions using optimized conditions also

15   yields the unligated fluorophore-conjugate that fluoresces, shown in **Figure 15, Panel C**. The total amount of fluorescent emission is therefore related to the amount of fluorophore-conjugate that is unligated. The efficiency of the ligation reaction then can be determined by monitoring the incorporation of the fluorescent unligated conjugate into the quenched ligated MB. incorporation is detected as a reduction in fluorescent

20   emission.

Advantages of this approach are several. First, optimization of ligation conditions permits product yields to be maximized. Second, maximizing product yields reduces the amount of unused label from each reaction. Third, monitoring MB synthesis reactions by melting and annealing reactions can be used to identify molecular beacons

25   that have optimal loop sequences. Fourth, the identification of optimal loop sequences within MBs can be used in the overall design of molecular beacons.

MONITORING MB SYNTHESIS IN A TEMPLATE-MEDIATED LIGATION REACTION

The reagents and reactions illustrated in **Figures 8-13** show a method for

30   the modular assembly of MBs by enzymatic ligation. The yield from ligation-based MB synthesis is directly related to the efficiency of the ligation reaction. As the efficiency of the reaction increases, the overall yield also increases. Monitoring the efficiency of each MB synthesis reaction can be done to optimize conditions for each enzymatic ligation. Discussed below is a method for directly monitoring ligation-based MB syntheses.

35   Illustrated in **Figure 16, Panel A** are three annealed oligonucleotides labeled Oligo D, Oligo E, and Oligo F. A full-length molecular beacon is synthesized by enzymatic ligation of the annealed component oligonucleotides, as shown in **Figure 16,**

5   **Panel B.** As the efficiency of the ligation reaction increases, the percentage of the ligated

       product shown in **Figure 16, Panel B** also increases, and the percentage of unligated

       component molecules shown in **Figure 16, Panel C** decreases. The annealing is

       performed at an optimal stringency to provide for specific self-annealing of the MB.

                        Melting and annealing of enzymatic ligation reactions using optimized

10   conditions yield the ligated MB that is quenched, shown in **Figure 16, Panel B**. Melting

       and annealing of enzymatic ligation reactions using optimized conditions also yield the

       unligated fluorophore-conjugate that fluoresces, shown in **Figure 16, Panel C**. The total

       amount of fluorescent emission is therefore related to the amount of fluorophore-

       conjugate that is unligated. The efficiency of the ligation reaction then can be determined

15   by monitoring the consumption of the fluorescent unligated conjugate into the quenched

       ligated MB. Consumption is detected as a reduction in fluorescent emission.

                        Advantages of this approach are also several. First, optimization of

       ligation conditions permits product yields to be maximized. Second, maximizing product

       amounts/yields reduces the amount of unused label from each reaction. Third, monitoring

20   MB synthesis reactions by monitoring melting and annealing reactions can be used to

       identify molecular beacons that have optimal loop sequences. Fourth, the identification

       of optimal loop sequences within MBs can be used in the overall design of molecular

       beacons.

       ## DETECTION OF COMPOSITE JUNCTION SEQUENCES

25   In a third class of embodiments which utilizes multiple templates for MB

       assembly, a loop structure is assembled that is comprised of a central region containing

       template ligation sequence, and two (or more) flanking regions containing sequences

       complementary to composite junction sequences. This class of embodiments is illustrated

       in **Figures 17-29**, involving the steps described therein and below. The sequences shown

30   in the Figures are meant to be for illustration of the principle only, not to limit the

       application to the illustrated examples. Any sequences which satisfy basic

       complementarity principles can be substituted. Further, any sequence that is a composite

       of two or more sequences can be usefully detected by the methods of this example.

                        A first short Oligonucleotide is synthesized with a fluorophore molecule

35   attached to its 5' end, by conventional methods of DNA synthesis as known by those

       skilled in the art ("Oligo D," *see*, **Figure 17**).

5   A second Oligonucleotide is synthesized with a quencher attached to its 3' end, and also bearing a 5' phosphate group, by conventional methods of DNA synthesis as known by those skilled in the art ("Oligo H," *see*, **Figure 18**). This second Oligo would also include a target-specific sequence which would become part of the "loop" (underline) of the molecular beacon, and a sequence for template-dependent ligation

10   (double underline).

          A third Oligonucleotide is synthesized bearing a 5' phosphate group by conventional methods of DNA synthesis ("Oligo G," *see*, **Figure 19**). This third Oligo includes a target-specific sequence which becomes part of a "loop" (underlined sequence) of the molecular beacon, and a sequence for template-dependent ligation (double

15   underline).

          Oligonucleotide G is designed such that it is complementary to sequences 5' to a splice junction that results from an alternative splicing event that joins exons 1 and 3, as illustrated in **Figure 20**.

          Oligonucleotide H is designed such that it is complementary to sequences

20   3' to a splice junction that results from an alternative splicing event that joins exons 1 and 3, as illustrated in **Figure 21**.

          A fourth Oligonucleotide is synthesized which bears a blocked 3' end, by conventional methods of DNA synthesis ("Oligo F," *see*, **Figure 22**). Oligo F is also designed to be complementary to both Oligo D and Oligo G, such that the three

25   Oligonucleotides together form the hybridized structure illustrated in **Figure 23**, in which a junction between the two double-stranded regions positions the 5' phosphate group of Oligo G immediately adjacent to the 3' hydroxyl group of Oligo D. In this orientation, Oligo D and Oligo G can be ligated into a single MB component. The 3' blockage of Oligo F can be achieved by various methods, such as the addition of a 3' phosphate group

30   or the use of a 3' dideoxynucleotide. The functional requirement of such a blockage is merely that it prevents Oligo F from taking part in any ligation reactions. Oligo F does not carry a 5' phosphate group, and therefore will not take part in ligation reactions at its 5' end.

          A fifth Oligonucleotide is synthesized which typically bears a blocked 3'

35   end (e.g., by incorporation of a dideoxy oligo at the 3' end), by conventional methods of DNA synthesis ("Oligo I," *see*, **Figure 24**). Oligo I is also designed to be complementary to both Oligo G and Oligo H, such that the three Oligonucleotides together form the

5   hybridized structure illustrated in **Figure 25**, in which a junction between the two double-
stranded regions positions the 5' phosphate group of Oligo H immediately adjacent to the
3' hydroxyl group of Oligo G. In this orientation, Oligo G and Oligo H can be ligated
into a single MB component. The 3' blockage of Oligo I can be achieved by various
methods, such as the addition of a 3' phosphate group or the use of a 3'

10 dideoxynucleotide. The functional reason for the blockage is merely that it prevent Oligo
I from taking part in any ligation reactions. Oligo I does not carry a 5' phosphate group,
and therefore will not take part in ligation reactions at its 5' end.

Oligonucleotides D, F, G, H, and I are annealed in a single reaction to
generate the structure shown in **Figure 26**.

15 Enzymatic ligation, using a enzyme such as T4 ligase or *E. coli* ligase or
any other enzyme capable of ligating nicks in double-stranded DNA molecule, is then
used to convert the hybridized structure composed of Oligo D, Oligo F, Oligo G, Oligo H,
and Oligo I into three Oligonucleotides (MB, D, G, H and the "ligation template Oligos"
F and I), as shown in **Figure 27**. Oligo D and Oligo H are designed such that they form

20 a complementary hybrid at their 5' (Oligo D) and 3' (Oligo H) ends, which form the stem
of the MB, as illustrated in **Figure 27** in such a way that the fluorophore and quencher
molecule are situated at the blunt end of the double-stranded stem to minimize the
distance between the two molecules and thereby maximize the quenching of the
fluorophore by the quencher. Temperatures and salt concentrations of the ligation

25 reaction are maintained such that the complementary overhangs are kept in close
proximity. Conditions of ligation can be manipulated in various ways known to those
skilled in the art for maximizing the efficiency of the reaction.

The ligated structure is purified away from the individual unligated
components. The ligated structure is also purified away from the excess of Oligos F and

30 I, which is easily achieved due to its greater length. Purification can be achieved either
by reverse-phase HPLC, or by any other available method, including, e.g., gel filtration,
ion exchange chromatography, or by other means known to those skilled in the art.

The full-length molecular beacon can be used to identify any two nucleic
acid sequences that are joined together, whether they be natural or synthetic, by

35 mechanisms that include splicing (e.g., as in RNA splicing), ligation (e.g., as in cloning),
PCR, hybridization, annealing, polymerization or by other means known to those skilled
in the art.

5   **Figure 28** illustrates one example of detection of juxtaposed sequences.
The example relates to detection of two alternative splicing events, A and B, that generate
two different junctions. Splicing reaction A joins exons 1, 2 and 3. Splicing reaction B
joins exons 1 and 3.

           Illustrated in **Figure 29** is the annealing of a MB to a target sequence after
10   melting and annealing using optimized temperatures and salt concentrations. The
annealing is performed at an optimal stringency to provide for the specific annealing of
the MB to both exons. The annealed MB results in a fluorescent emission. The
conditions can be manipulated in various ways known to those skilled in the art for
maximizing the efficiency of the reaction. The target is composed of sequence from the
15   3' end of exon 1 and sequence from the 5' end of exon 3. The MB anneals to both exon 1
and exon 3 target sequences, generating a central loop that contains unhybridized
template ligation sequence.

           This example is shown to illustrate that MBs containing sequences
complementary to two (or, optionally, more) different target sequences can be used to
20   identify splicing variants, or any other composite nucleic acid sequences that are
complementary to the sequences flanking the template ligation sequence. These include
cloned sequences, ligated sequences, spliced sequences and any others known in the art.

           Advantages of this approach include the following. First, modular
synthesis of MBs can be used to join any two (or more) combinations of sequences
25   together without the need to re-synthesize entire MBs from scratch for each potential
target to be detected. Second, synthesis of MBs with two (or more) regions of target
sequence complementarity permits the identification of specific splice variants for any
given gene. Third, syntheses of MBs with two regions of target sequence
complementarity permits the detection of vector and insert sequences at junctions in
30   cloning reactions.

MONTIORING REACTIONS

           In the methods noted above, the efficiency of ligation reactions is
monitored by measuring fluorescence or other relevant MB label properties. This can be
performed by any available method e.g., spectrophotometrically, optically, via
35   microscopy, or the like. Common detectors appropriate to such methods include
spectrophotometers, CCD arrays, photo multiplier tubes, photodiodes, scanning detectors,

22

5   microscopes, galvo-scanns and the like. The detector can interface with the system or

device for detection regardless of what fluid handling system is used to perform the

ligation reactions. Thus, whether the reactions are performed in test tubes, cuvettes,

microtiter trays or in microscale systems, detectors are commonly available that can be

used to detect photon (or other) signal properties for a reaction system. Devices/ systems

10   of the invention can include a detector proximal to a ligation reaction region, or proximal

to a hybridization reaction region of the device. Such reaction/ hybridization regions

include high-throughput reaction regions such as microtiter trays (e.g., 96, 384 or more

well trays), container handling systems (e.g., which robotically handle trays, test tubes, or

the like), microfluidic devices, or the like. The detection system is typically coupled to a

15   computer, optionally via an analog to digital or digital to analog converter, for

transmitting detected signal data to a computer for analysis, storage and data

manipulation.

In one aspect, the invention provides a device, e.g., which includes a

reaction region and a ligation mixture as described herein. The reaction region is

20   commonly part of a high-throughput fluid handling system, such as a microtiter tray or a

microfluidic component. The device optionally includes a detector proximal to the

reaction region, i.e., any detector as noted above. The device further optionally includes

robotics for moving microtiter trays or other fluid containers, one or more computers for

controlling the device or receiving and analyzing data from the detector, or the like.

25   **Figure 30** schematically depicts such a device, i.e., having detector **30** proximal to

microtiter tray **40**. The device/ system is shown operably coupled to computer **50** and

having robotic armature **50** for manipulating tray **40**. Ligation or hybridization mixtures

are formed in tray **40** in this embodiment.

A variety of configurations of these basic elements can be used to form a

30   system or device of the invention.

MB COMPONENT SYNTHESIS

MB components (oligos, including those labeled with fluorophores or

quenchers) can be synthesized using conventional methods. For example, oligos or PNAs

can be synthesized on commercially available automated oligonucleotide/PNA synthesis

35   machines using standard methods. Labels can be attached to the oligos or PNAs either

during automated synthesis or by post-synthetic reactions which have been described

5   before *see, e.g.,* Tyagi and Kramer (1996) "Molecular beacons: probes that fluoresce
     upon hybridization" Nature Biotechnology 14:303-308 and USP 6,037,130 to Tyagi et al
     (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in
     assays and kits." and U.S. Pat. No. 5,925,517 (July 20, 1999) to Tyagi et al. entitled
     "Detectably labeled dual conformation oligonucleotide probes, assays and kits."

10   Additional details on synthesis of functionalized oligos can be found in Nelson, et al.
     (1989) "Bifunctional Oligonucleotide Probes Synthesized Using A Novel CPG Support
     Are Able To Detect Single Base Pair Mutations" Nucleic Acids Research 17:7187-7194.
            Labels/ quenchers can be introduced to the oligonucleotides or PNAs, e.g.,
     by using a controlled-pore glass column to introduce, e.g., the quencher (e.g., a 4-

15   dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL). For example, the quencher
     can be added at the 3' end of oligonucleotides during automated synthesis; a succinimidyl
     ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) can be used when the
     site of attachment is a primary amino group; and 4-dimethylaminophenylazophenyl-4'-
     maleimide (DABMI) can be used when the site of attachment is a sulphydryl group.

20   Similarly, fluorescein can be introduced in the oligos, either using a fluorescein
     phosphoramadite that replaces a nucleoside with fluorescein, or by using a fluorescein dT
     phosphoramadite that introduces a fluorescein moiety at a thymidine ring via a spacer.
     To link a fluorescein moiety to a terminal location, iodoacetoamidofluorescein can be
     coupled to a sulphydryl group. Tetrachlorofluorescein (TET) can be introduced during

25   automated synthesis using a 5'-tetrachloro-fluorescein phosphoramadite. Other reactive
     fluorophore derivatives and their respective sites of attachment include the succinimidyl
     ester of 5-carboxyrhodamine-6G (RHD) coupled to an amino group; an iodoacetamide of
     tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of
     tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red

30   coupled to a sulphydryl group. During the synthesis of these labeled components,
     conjugated oligonucleotides or PNAs can be purified, if desired, e.g., by high pressure
     liquid chromatography or other methods.
            In general, synthetic methods for making oligonucleotides and PNAs
     (including labeled oligos and PNAs) is well known. For example, oligonucleotides can

35   be synthesized chemically according to the solid phase phosphoramidite triester method
     described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20):1859-1862, e.g.,
     using a commercially available automated synthesizer, e.g., as described in Needham-

5   VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168. Oligonucleotides,

       including modified oligonucleotides can also be ordered from a variety of commercial

       sources known to persons of skill. There are many commercial providers of oligo

       synthesis services, and thus, this is a broadly accessible technology. Any nucleic acid can

       be custom ordered from any of a variety of commercial sources, such as The Midland

10   Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company

       (www.genco.com), ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc.

       (Alameda, CA) and many others. Similarly, PNAs can be custom ordered from any of a

       variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc.

       (www.htibio.com), BMA Biomedicals Ltd (U.K.), Bio-Synthesis, Inc., and many others.

15   <u>LIGATION OF OLIGONUCLEOTIDES AND PNAS</u>

              A number of approaches are available for the ligation of oligonucleotides,

       PNAs and even for ligation of PNAs to oligonucleotides. Thus, the MBs of the invention

       can be formed from PNAs, oligonucleotides, or both PNAs and oligonucleotides.

       Ligation, as used in this context, simply refers to producing a covalent bond between the

20   two or more elements to be ligated.

              Ligation of nucleic acids by enzymatic approaches is well known. The

       basic enzymes and reaction conditions for ligation can be found in any of a variety texts,

       including, e.g., Berger and Kimmel (1989), <u>Guide to Molecular Cloning Techniques,</u>

       <u>Methods in Enzymology</u> volume 152 Academic Press, Inc., San Diego, CA (Berger);

25   Sambrook et al., <u>Molecular Cloning - A Laboratory Manual</u> (2nd Ed.), Vol. 1-3, Cold

       Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook") and

       <u>Current Protocols in Molecular Biology</u>, F.M. Ausubel et al., eds., Current Protocols, a

       joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc.,

       (supplemented through 2000) ("Ausubel")). Oligonucleotide-PNA chimeras can also be

30   made. *See*, Koppitz, M., Nielsen P.E. & Orgel, L. (1997) Formation of oligonucleotide-

       PNA-Chimeras by Template-directed Ligation. J. Amer. Chem. Soc. 120, 4563-4569.

              Ligation of nucleic acids via chemical approaches also uses any of a

       variety of well known chemistries. Chemical ligation can involve use of reactive groups

       on the molecules to be ligated, such as hydroxyl, phosphate, sulfhydryl, amino, alkyl

35   phosphate, alkyl amino or hydroxy alkyl groups. Covalently reactive groups are

       preferred. Examples of the basic chemistry relevant to chemical ligation include Stryer

5 (1988) <u>Biochemistry</u>, third edition (or later editions) Freeman and Co. New York, NY;

Pine *et al.* <u>Organic Chemistry Fourth Edition</u> (1980) McGraw-Hill, Inc. (USA) (or later

editions); March, <u>Advanced Organic Chemistry Reactions, Mechanisms and Structure</u> 4th

ed J. Wiley and Sons (New York, NY, 1992) (or later editions); Greene, et al., <u>Protective</u>

<u>Groups In Organic Chemistry</u>, 2nd Ed., John Wiley & Sons, New York, NY, 1991 (or

10 later editions); Lide (ed) (1995) <u>The CRC Handbook of Chemistry and Physics 75th</u>

<u>edition</u> (or later editions); and in the references cited in the foregoing.

        Chemistries for joining short oligonucleotides (e.g., trinucleotides) are

especially well known and can be adapted to the present invention to chemically join

longer oligonucleotides. References that show chemical linkage of short oligos include

15 Virnekäs, B., *et al.*, (1994) <u>Nucleic Acids Res.</u>, 22, 5600-5607; Kayushin, A. L. *et al.*,

(1996) <u>Nucleic Acids Res.</u>, 24, 3748-3755, Huse, USP 5,264,563 "Process For

Synthesizing Oligonucleotides With Random Codons," Lyttle *et al.*, USP 5,717,085

"Process For Preparing Codon Amidites," Shortle *et al.*, USP 5,869,644 "Synthesis Of

Diverse And Useful Collections Of Oligonucleotides," Greyson, USP 5,789,577 "Method

20 For The Controlled Synthesis Of Polynucleotide Mixtures Which Encode Desired

Mixtures Of Peptides," and Huse, WO 92/06176 "Surface Expression Libraries Of

Randomized Peptides."

DONORS AND ACCEPTORS (FLUOROPHORES AND QUENCHERS) IN MBS

        In MBs, a central target-recognition sequence is flanked by arms that

25 hybridize to one another when the probe is not hybridized to a target strand, forming a

"hairpin" structure, in which the target-recognition sequence (which is sometimes referred

to as the "probe sequence") is in the single-stranded loop of the hairpin structure, and the

arm sequences form a double-stranded stem hybrid.

        Operation of the MB is rather straightforward. When the probe hybridizes

30 to a target, a relatively rigid helix is formed, causing the stem hybrid to unwind and

forcing the arms of the MB apart. A label/ quencher pair, such as the fluorophore

EDANS and the quencher DABCYL, are attached to the arms, e.g., by alkyl spacers.

When the MB is not hybridized to a target strand, the fluorophore's emission is quenched.

When the Molecular Beacon is hybridized to a target strand, the FRET pair is separated

35 by more than 100 angstroms, and the fluorophore's emission is not quenched. Thus,

5   emitted fluorescence signals the presence, in real time, of target strands being hybridized
       to the MB.

              Molecular beacon probes can have target recognition sequences of
       typically about 7-140 nucleotides in length and arms that form a stem hybrid, or "stem
       duplex" of about 3-25 nucleotides in length. Modified nucleotides and modified
10   nucleotide linkages may be used for MB construction, even including, e.g., peptide
       nucleic acid ("PNAs").

              <u>FRET Probes</u>
              As noted, MBs made according to the present invention can utilize FRET
       to detect nucleic acid hybridization. Fluorescence resonance energy transfer (FRET) is a
15   distance-dependent interaction between the electronic excited states of two molecules
       (e.g., a fluorophore and a quenching molecule) in which excitation is transferred from a
       donor molecule (the fluorophore in this example) to an acceptor molecule (the quenching
       molecule in this example) typically without any photon emission. FRET is dependent on
       an inverse sixth power of the separation of the donor and acceptor moieties, making it
20   useful over distances comparable with the dimensions of biological macromolecules.

              The primary conditions for typical FRET are well known and can be used
       in the design and construction of MBs of the invention. First, donor and acceptor
       molecules are in close proximity (typically 10–100 Å) for FRET to occur. Second, the
       absorption spectrum of the acceptor typically overlaps the fluorescence emission
25   spectrum of the donor. Third, donor and acceptor transition dipole orientations are
       usually approximately parallel.

              As noted, MBs can incorporate any of a variety of fluorophore/ quencher
       (in this context, "donor/ acceptor") combinations. With regard to such pairs, there are a
       number of fluorophores which are known to quench one another. Examples of interactive
30   fluorescent label pairs include terbium chelate and TRITC (tetrarhodamine
       isothiocyanate), europium cryptate and Allophycocyanin, Fluorescein and
       Tetramethylrhodamine, IAEDANS and Fluorescein, EDANS and DABCYL, Fluorescein
       and Fluorescein, BODIPY FL and BODIPY FL, and Fluorescein and QSY 7 dye.

              Fluorescence quenching is a bimolecular process that reduces the
35   fluorescence quantum yield, typically without changing the fluorescence emission
       spectrum. Quenching can result from transient excited state interactions, (collisional
       quenching) or, e.g., from the formation of nonfluorescent ground state species. Self

5   quenching is the quenching of one fluorophore by another; it tends to occur when high

concentrations, labeling densities, or proximity of labels occurs. Fluorescent resonance

energy transfer (FRET) is a distance dependent excited state interaction in which

emission of one fluorophore is coupled to the excitation of another which is in proximity

(close enough for an observable change in emissions to occur).

10   In some applications, the donor and acceptor moieties are different, in

which case MB hybridization is detected by the appearance of sensitized fluorescence of

the acceptor (or even by quenching of donor fluorescence, i.e., in the context of the

invention by measuring elimination of fluorescence by elimination of target-specific

hybridization). When the donor and acceptor are the same, FRET can be detected by

15   fluorescence depolarization.

              Nonfluorescent acceptors such as DABCYL and QSY 7 and QSY 33 dyes

have the particular advantage of eliminating background fluorescence resulting from

direct (i.e., nonsensitized) acceptor excitation. A variety of probes incorporating

fluorescent donor–nonfluorescent acceptor combinations have been developed for

20   detection of nucleic acid hybridization events. *See also*, Haugland (1996) <u>Handbook of</u>

<u>Fluorescent Probes and Research Chemicals</u> Published by Molecular Probes, Inc.,

Eugene, OR. e.g., at chapter 13) or a more current on-line (www.probes.com) or CD-

ROM version of the Handbook (available from Molecular Probes, Inc.).

<u>Non-FRET Based Quenchers in MBs</u>

25   An alternate to the use of FRET probes in the context of MBs is found in

USP 6,150,097 to Tyagi et al (November 21, 2000) entitled "Nucleic acid detection

probes having non-FRET fluorescence quenching and kits and assays including such

probes." Instead of using donor-acceptor moieties that follow the typical rules of FRET,

MBs can bring the fluorophore and quencher into very close contact, resulting in

30   quenching regardless of whether FRET occurs. That is, when attached to a MB such that

it is in contact with, or "touching", the first fluorophore in one of its conformations, a

quenching moiety need not have an absorption spectrum that overlaps the emission

spectrum of the first fluorophore. Furthermore, the absorption wavelength of the

quencher can be shorter than the fluorophore's excitation maximum and emission

35   wavelength. Similarly, a second fluorophore that absorbs at a wavelength shorter than the

emission wavelength of the first can, in the probe construction described above, act as a

5   quencher; that is, suppress emission by the first fluorophore and dissipate the incident

energy as heat rather than as photon emission.

In probes constructed according to these methods, changes in the

absorption spectra of the label pair can be used as a detectable signal, rather than simply

monitoring a change in fluorescence. When a change in absorption is utilized, the label

10   pair may include any two chromophores, that is, fluorophores, quenchers and other

chromophores. The label pair can even include identical chromophores or fluorophores.

Thus, for example, DABCYL, when attached to one end of a Molecular Beacon, can

effectively quench fluorophores attached to the other end of the MB. This is in violation

of standard FRET rules, enlarging the available number of fluorophore-quencher pairs

15   that can be used in MBs beyond those available for FRET.

LIBRARIES OF MB COMPONENTS AND LIGATION MIXTURES

One aspect of the invention is the construction of libraries of MB

components which can be ligated according to the methods herein. The libraries include

a set of a hairpin loop oligonucleotides or PNAs, each of which includes a subsequence of

20   at least one molecular beacon (but less than all of the final product MB). These oligos are

typically arrayed or stored in an otherwise accessible format. For example, the oligos can

be arrayed on a microtiter tray. Other components can be added to the array members,

including the addition of at least one label or label quenching oligonucleotide or PNA

(i.e., an oligonucleotide which includes a label or a label quencher). Ligation of at least

25   one hairpin oligonucleotide or PNA and the label or label quenching oligonucleotide or

PNA produces a molecular beacon or molecular beacon subsequence—and thus an array

of MBs.

In one embodiment, the array of MBs includes oligos which are

systematically varied with respect to the loop or stem regions, i.e., each differing by one

30   or a few monomer units (nucleotides, PNA monomers, etc.). MB synthesis and

hybridization properties are monitored in real time, facilitating selection of sequences

with optimal MB synthesis properties, and/or having optimal hybridization properties for

one or more target.

In other embodiments, the arrays are designed to optimize other MB

35   parameters such as multiplexing capabilities. Multiplexed detection schemes (e.g.,

simultaneously monitoring different hybridization events by monitoring different labels in

29

5   a single solution) are of increasing value generally. Thus, the present invention's ability

to optimize properties in an array based format provides a mechanism for simultaneously

optimizing one or more than one variable across a population of MBs or MB synthesis

schemes.

As noted, members of the library can be located in a microtiter tray or

10   other array, with individual members types of the hairpin loop oligonucleotides or PNAs

being located in wells of the microtiter tray. The wells (or other array containing

compartments), can further include components relevant to MB synthesis or activity,

including ligases, ligation buffers, the label or label quenching oligonucleotide or PNA,

target nucleic acids, or the like.

15   SYSTEMS COMPRISING OLIGOS, LIGATION COMPONENTS AND SEQUENCE
INFORMATION

In one aspect, the invention includes systems for the design and synthesis

of the MBs according to the methods herein. The system can include, e.g., a computer

with appropriate software for manipulating text strings corresponding to oligos or PNAs

20   to be synthesized, software for modeling MB hybridization to a target, etc. Similarly, the

system can include an automated synthesizer coupled to the computer (directly or

indirectly, e.g., through a cable, LAN or across the internet) for synthesizing oligos

corresponding to the character strings in the computer.

The computer typically includes appropriate software for receiving user

25   instructions, either in the form of user input into a set parameter fields, e.g., in a GUI, or

in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different

specific operations. The software optionally converts these instructions to appropriate

language for instructing the operation of the synthesizer to carry out the desired synthetic

operation. The computer can also receives data from the synthesizer regarding yield,

30   cycle completion or the like and can interpret the data, provide it to a user in a human

readable format, or can use that data to initiate further synthetic operations, in accordance

with any programming by the user.

KITS

The present invention can include kits that include any of the components

35   of the compositions, systems, ligation mixtures or libraries herein. Typically, the kits

further include features that are relevant to the distribution of such materials, including

30

5   packaging materials, instructions for using the components to produce one or more
molecular beacons, one or more containers for holding the components, or the like.

In addition, components providing positive or negative controls for any
reaction relevant to the synthesis or use of a molecular beacon can be provided. These
include standards for calibrating any MB synthesis or detection reaction, standard target

10   sequences (e.g., positive or negative controls that are known to bind or to not bind to the
MB at issue), or the like. In addition, auxiliary components relevant to any MB detection
reaction can be included, such as amplification primers for amplifying a target sequence
(e.g., via PCR or LCR) can be included.

While the foregoing invention has been described in some detail for

15   purposes of clarity and understanding, it will be clear to one skilled in the art from a
reading of this disclosure that various changes in form and detail can be made without
departing from the true scope of the invention. For example, all the techniques and
compositions described above may be used in various combinations. All publications,
patent applications, patents and/or other documents cited in this application are

20   incorporated by reference in their entirety for all purposes to the same extent as if each
individual publication or patent document were individually so denoted.

5   WHAT IS CLAIMED IS:

1. A method of making one or more molecular beacons or molecular beacon components, comprising:

providing a first oligonucleotide or peptide nucleic acid (PNA) corresponding to a first subsequence of a molecular beacon;

10   providing a second oligonucleotide or PNA corresponding to a second subsequence of a molecular beacon;

ligating the first and second oligonucleotides or PNAs together, thereby forming the molecular beacon or the molecular beacon component; and,

monitoring a ligation-dependent change in a signal output of the molecular

15   beacon, the molecular beacon component, the first oligonucleotide or PNA, or the second oligonucleotide or PNA.

2. The method of claim 1, wherein the ligation-dependent signal output is a change in a fluorescence emission at a hybridization temperature that permits intra-molecular hybridization of the molecular beacon.

20   3. The method of claim 1, further comprising optimizing one or more reaction parameters to increase yield of the molecular beacon or molecular beacon component or efficiency of the ligating step.

4. The method of claim 1, further comprising optimizing one or more reaction parameters to minimize an amount of unligated material remaining following the ligating

25   step.

5. The method of claim 1, further comprising monitoring one or more melting and annealing reactions by monitoring one or more intra-molecular melting or intra-molecular annealing dependent reactions.

6. The method of claim 5, comprising identifying one or more MB that has an

30   optimized loop sequence.

7. The method of claim 1, wherein the first oligonucleotide or PNA comprises a label moiety and the second oligonucleotide comprises a label quenching moiety.

5   8. The method of claim 7, wherein the label moiety is selected from Texas red, terbium chelate, europium cryptate, DABCYL, Fluorescein, IAEDANS, EDANS, and BODIPY FL.

9. The method of claim 7, wherein the quenching moiety is selected from TRITC (tetrarhodamine isothiocyanate), Allophycocyanin, EDANS, Tetramethylrhodamine,

10   DABCYL, Fluorescein, BODIPY FL, and QSY 7 dye.

10. The method of claim 1, further comprising ligating a third oligonucleotide or PNA to the first or second oligonucleotide, or to the molecular beacon component.

11. The method of claim 10, wherein the third oligonucleotide or PNA is ligated to the molecular beacon component, thereby providing the molecular beacon.

15   12. The method of claim 10, wherein the first, second and third oligonucleotides are ligated using two template oligonucleotides to align the first, second and third oligonucleotides for ligation.

13. The method of claim 10, wherein the first, second and third oligonucleotides are complementary to two or more proximal subsequences in a target nucleic acid.

20   14. The method of claim 10, wherein the first, second and third oligonucleotides are complementary to two or more proximal subsequences in a target nucleic acid, wherein the subsequences are made proximal in the target nucleic acid by splicing or ligating the subsequences together.

15. The method of claim 1, wherein the first and second oligonucleotides or PNAs

25   are aligned on a template nucleic acid prior to said ligating step.

16. The method of claim 15, wherein the template nucleic acid is a synthetic single-stranded oligonucleotide.

17. The method of claim 1, wherein the ligating step is performed via enzymatic ligation.

30   18. The method of claim 17, wherein the ligating step is performed using a ligase enzyme selected from: a Taq DNA ligase, an E. coli DNA ligase, and a T4 DNA ligase.

19. The method of claim 1, wherein the ligating step is performed via chemical ligation.

5   20. The method of claim 1, further comprising purifying the molecular beacon
from one or more unligated first or second oligonucleotides or PNAs.

           21. The method of claim 20, wherein the molecular beacon is purified from the
unligated first or second oligonucleotides or PNAs using HPLC.

           22. A device comprising:

10   a reaction region comprising a ligation mixture therein, the ligation mixture
comprising a first oligonucleotide or PNA comprising a label moiety; a second
oligonucleotide or PNA comprising a quenching moiety that quenches the label moiety
when placed proximal to or in contact with the label moiety; a third oligonucleotide or
PNA that is at least partly complementary to at least a portion of the first and second

15   oligonucleotides; and, a ligase; and,
           a detector proximal to the reaction region, which detector detects one or more
ligation-dependent change in an output of the label moiety.

           23. The device of claim 22, wherein ligation of the first, second and third
oligonucleotides or PNAs results in formation of a molecular beacon.

20   24. The device of claim 22, wherein the first and second oligonucleotides or
PNAs are at least partly complementary.

           25. The device of claim 22, wherein ligation of the first and second
oligonucleotides or PNAs results in formation of a molecular beacon and wherein the
third oligonucleotide provides a template for ligation of the first and second

25   oligonucleotides.

           26. The device of claim 22, wherein ligation of the first, second and third
oligonucleotides or PNAs results in formation of a molecular beacon and wherein the
ligation mixture further comprises fourth and fifth oligonucleotides which provide a
template for ligation of the first, second and third oligonucleotides.

30   27. The device of claim 22, wherein, upon ligation, nucleotides of the first and
second oligonucleotides form at least a portion of a molecular beacon stem and
nucleotides of the third oligonucleotide forms at least portion of a hairpin loop portion of
the molecular beacon.

5   **28.** The device of claim **22**, wherein the label or quenching moiety is selected from Texas red, terbium chelate, europium cryptate, DABCYL, Fluorescein, IAEDANS, EDANS, and BODIPY FL.

           29. The device of claim **22**, wherein the label or quenching moiety is selected from TRITC (tetrarhodamine isothiocyanate), Allophycocyanin, EDANS,
10   Tetramethylrhodamine, DABCYL, Fluorescein, BODIPY FL, and QSY 7 dye.

           30. The device of claim **22**, wherein the ligase is selected from E. coli ligase, T4 ligase, and Taq ligase.

           31. The device of claim **22**, further comprising a ligation buffer.

           32. The device of claim **22**, wherein the second oligonucleotide or PNA is at least
15   partly complementary to one or more target nucleic acid.

           33. The device of claim **22**, wherein the second oligonucleotide or PNA is at least partly complementary to one or more single nucleotide polymorphism.

           34. A kit comprising each of the components of the ligation mixture of claim **22**.

           35. The kit of claim **34**, further comprising one or more of: packaging materials,
20   instructions for using the components to produce one or more molecular beacons, one or more containers for holding the components, standards for calibrating any MB detection reaction, standard target sequences, or amplification primers for amplifying a target sequence.

           36. A library of molecular beacon components, comprising:
25   a set of a plurality of hairpin loop oligonucleotides or PNAs, each of the plurality of hairpin loop oligonucleotides or PNAs comprising a subsequence of at least one molecular beacon, the subsequence comprising less than all of the molecular beacon; and,
           at least one label or label quenching oligonucleotide or PNA, which oligonucleotide or PNA comprises at least one label or label quenching moiety, wherein
30   ligation of at least one hairpin oligonucleotide or PNA and the label or label quenching oligonucleotide or PNA produces a molecular beacon or molecular beacon subsequence.

           37. The library of claim 36, wherein the hairpin loop oligonucleotide or PNA comprises a label or label quenching moiety, and wherein ligation of the hairpin loop

5   oligonucleotide or PNA to the label or label quenching oligonucleotide or PNA produces

a molecular beacon.

38. The library of claim 36, the library comprising both a label oligonucleotide or

PNA and a label quenching oligonucleotide or PNA, wherein ligation of the label

oligonucleotide or PNA, the label quenching oligonucleotide or PNA and the hairpin loop

10   oligonucleotide or PNA produces a molecular beacon.

39. The library of claim 36, wherein the library is located in a microtiter tray, with

individual members types of the hairpin loop oligonucleotides or PNAs being located in

wells of the microtiter tray.

40. The library of claim 36, wherein the library is located in a microtiter tray, with

15   individual members types of the hairpin loop oligonucleotides or PNAs being located in

wells of the microtiter tray, wherein each well of the tray further comprises one or more

of: a ligase, a ligation buffer, a target nucleic acid, or the label or label quenching

oligonucleotide or PNA.

41. The library of claim 36, further comprising one or more ligase enzyme.

20   42. A kit comprising the library of claim 36 and one or more of: packaging

materials, instructions for using the library to produce one or more molecular beacons,

one or more containers for holding one or more components of the library, one or more

ligase enzyme, one or more standard target molecule, one or more amplification

oligonucleotides, or one or more ligation buffer.

25   43. A method of detecting a juxtaposition of two or more target subsequences in a

target nucleic acid, comprising:

forming a molecular beacon by ligating a first oligonucleotide complementary to

the first target subsequence and a second oligonucleotide complementary to the second

target subsequence;

30   hybridizing the resulting molecular beacon to the target nucleic acid; and,

detecting target-specific hybridization of the molecular beacon to the first and

second subsequences.

44. The method of claim 43, wherein the first and second oligonucleotide are

ligated by aligning the oligonucleotides on one or more templates and incubating the

35   resulting hybridized set of oligonucleotides with a ligase.

5   **45.** The method of claim **43**, wherein forming the molecular beacon further

comprises ligating one or more additional oligonucleotides to the first and second

oligonucleotides, which one or more additional oligonucleotides comprise a label or a

label quenching moiety. .

         46. The method of claim **43**, wherein forming the molecular beacon further

10   comprises purifying the molecular beacon from an unligated first or second

oligonucleotide.

         47. The method of claim **43**, wherein the juxtaposed target subsequences are

juxtaposed by one or more of: RNA splicing, RNA splicing and reverse transcription,

ligation, or PCR.

Figure 1:
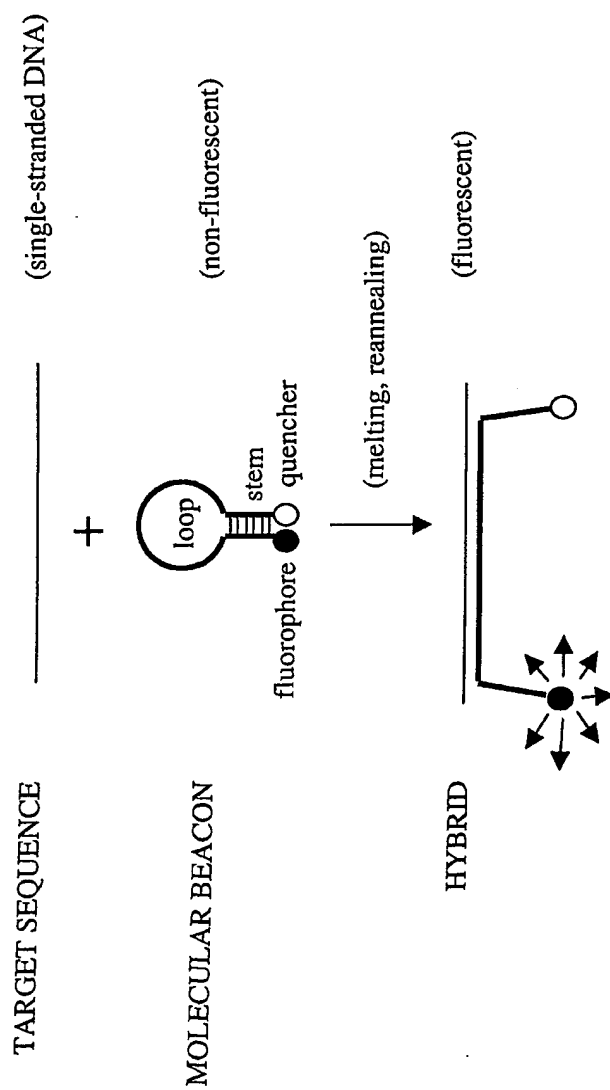
Fluorescein – 5'– CGTCTGCT 3'– OH

OR

Dabsyl – 5'– CGTCTGCT 3'– OH

(Oligo A)

Figure 2

Dabsyl $-3'-$GCAG$-5'-$PO$_4$

OR

Fluorescein $-3'-$GCAG$-5'-$PO$_4$

(Oligo B)

Figure 3

Figure 4

(Oligo A) Fluorescein – 5'– CGTCTGCT 3'– OH
(Oligo B) Dabsyl – 3'– GCAG 5'– PO$_4$

OR

(Oligo A) Dabsyl – 5'– CGTCTGCT 3'– OH
(Oligo B) Fluorescein – 3'– GCAG 5'– PO$_4$

$_4$OP-5²-CCCCAT
         | | | |
HO-3²-ACGAGGGGCT

(oligo C)

Figure 5

Figure 6

nick

Fluorescein – 5'– CGTCTGCTCCCAT
Dabsyl – 3'– GCAGACGAGGGGGCT

nick

(MB. Olgos A–C)
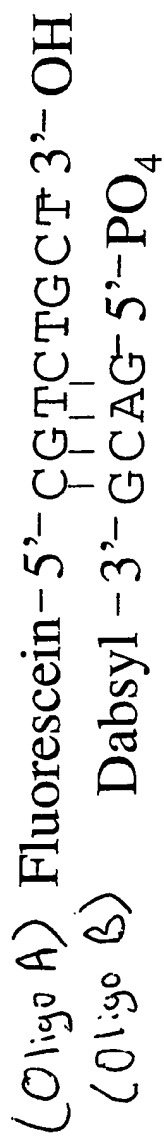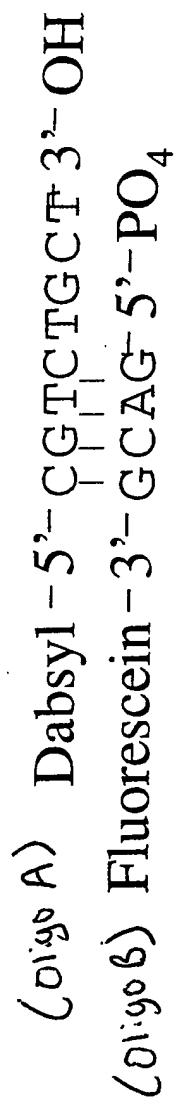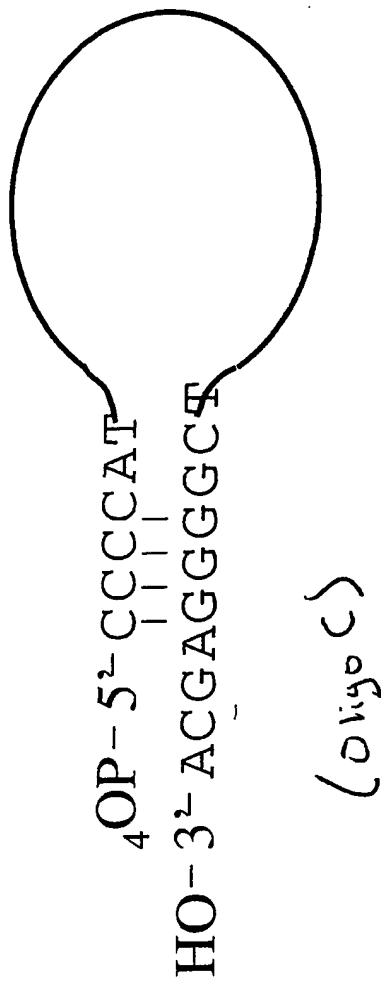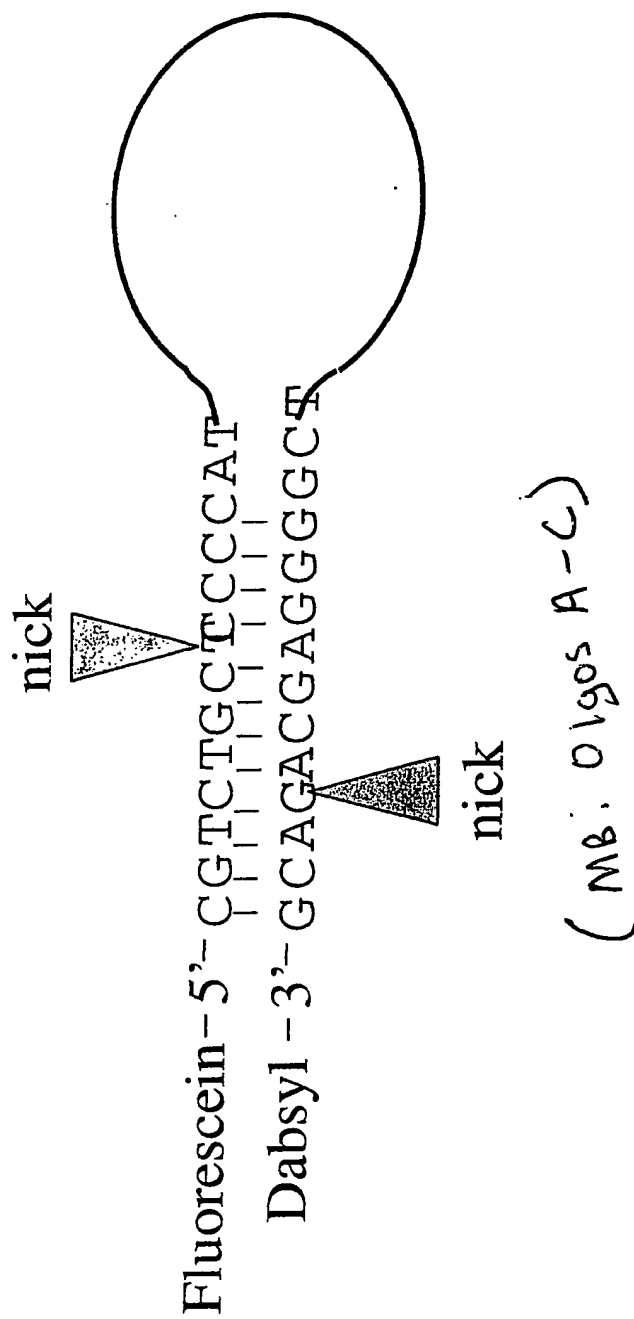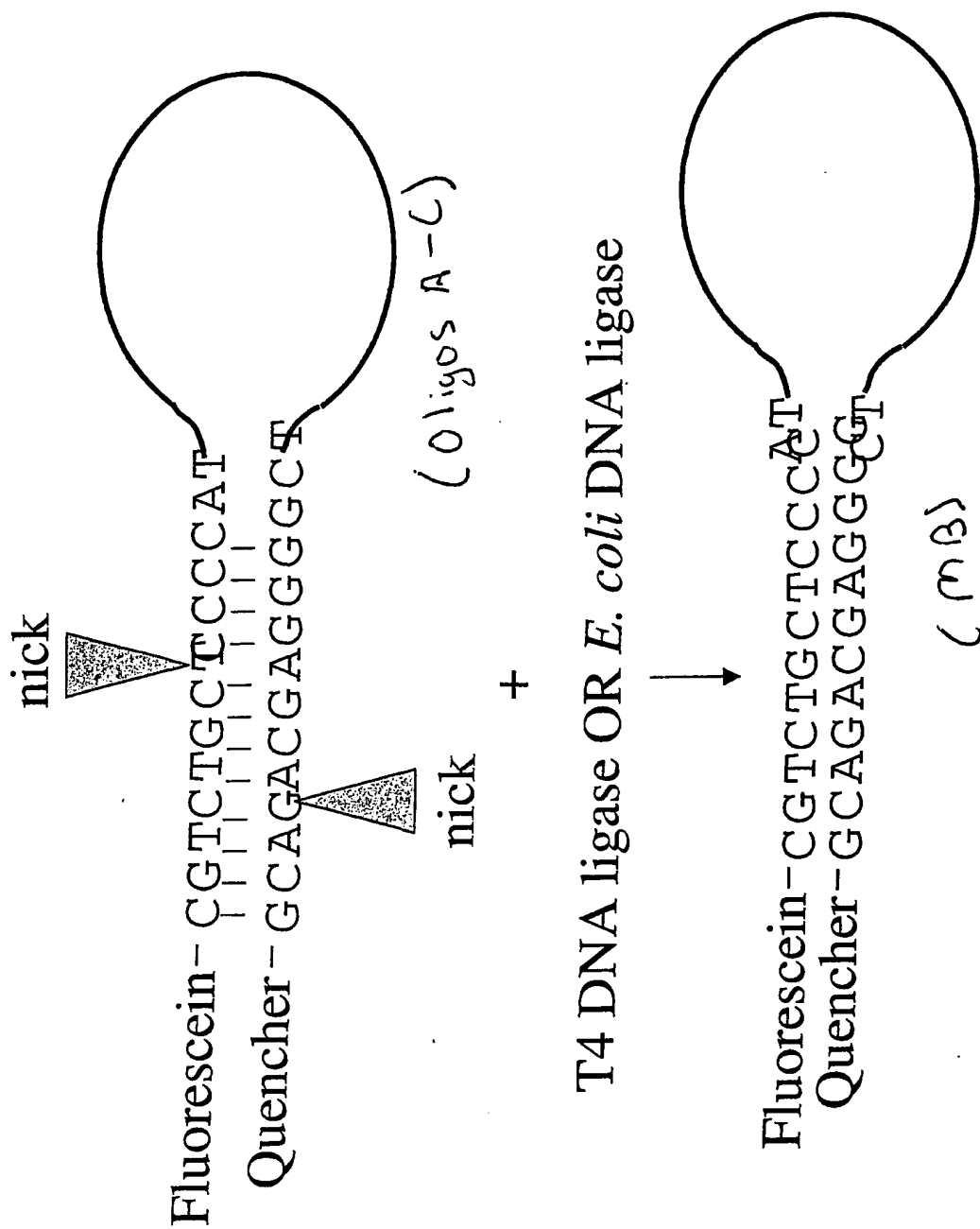
Figure 7:

F-5'-CGCCGC-3'-OH

(oligo D)

Figure 8

$$PO_4-5'\text{-CGACG}\text{---GCGGCG}-3'\text{-Q}$$

(Oligo E)

Figure 9

Figure 10

B –3′-GCGGCGGCTGC –5′

(Oligo F)

Figure 11

Oligo E

PO$_4$–5'–CGACG———GCGGCG–3'–Q

B–3'–GCGGGCGGGCTGC–5'

Oligo F

Oligo D

F–5'–CGCCGC–3'–OH

B–3'–GCGGGCGGGCTGC–5'

Oligo F

Figure 12

Oligo E

Oligo D

Oligo F

nick

F –5′–CGCCGCCGACG————GCGGCG –3′–Q
          |||||||||
B –3′–GCGGGGCTGC –5′
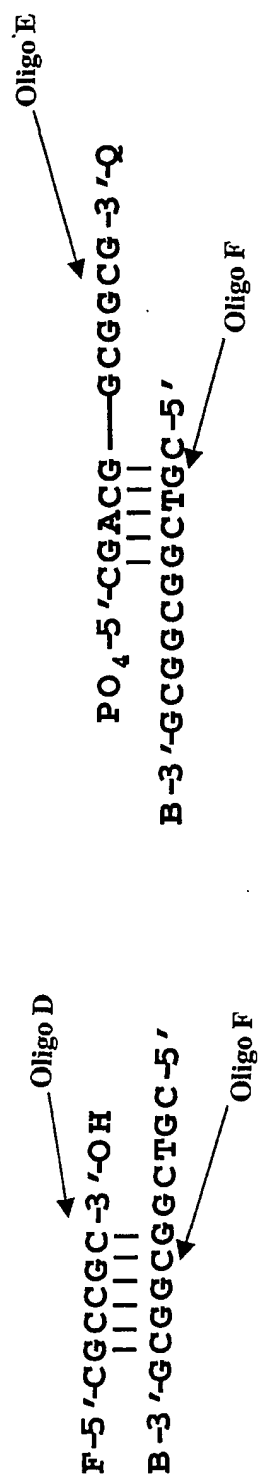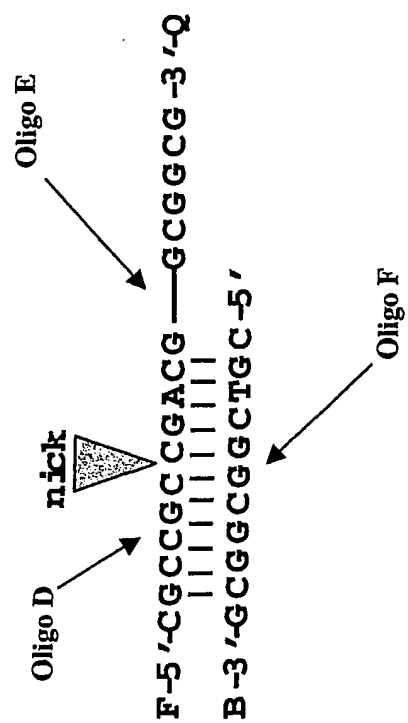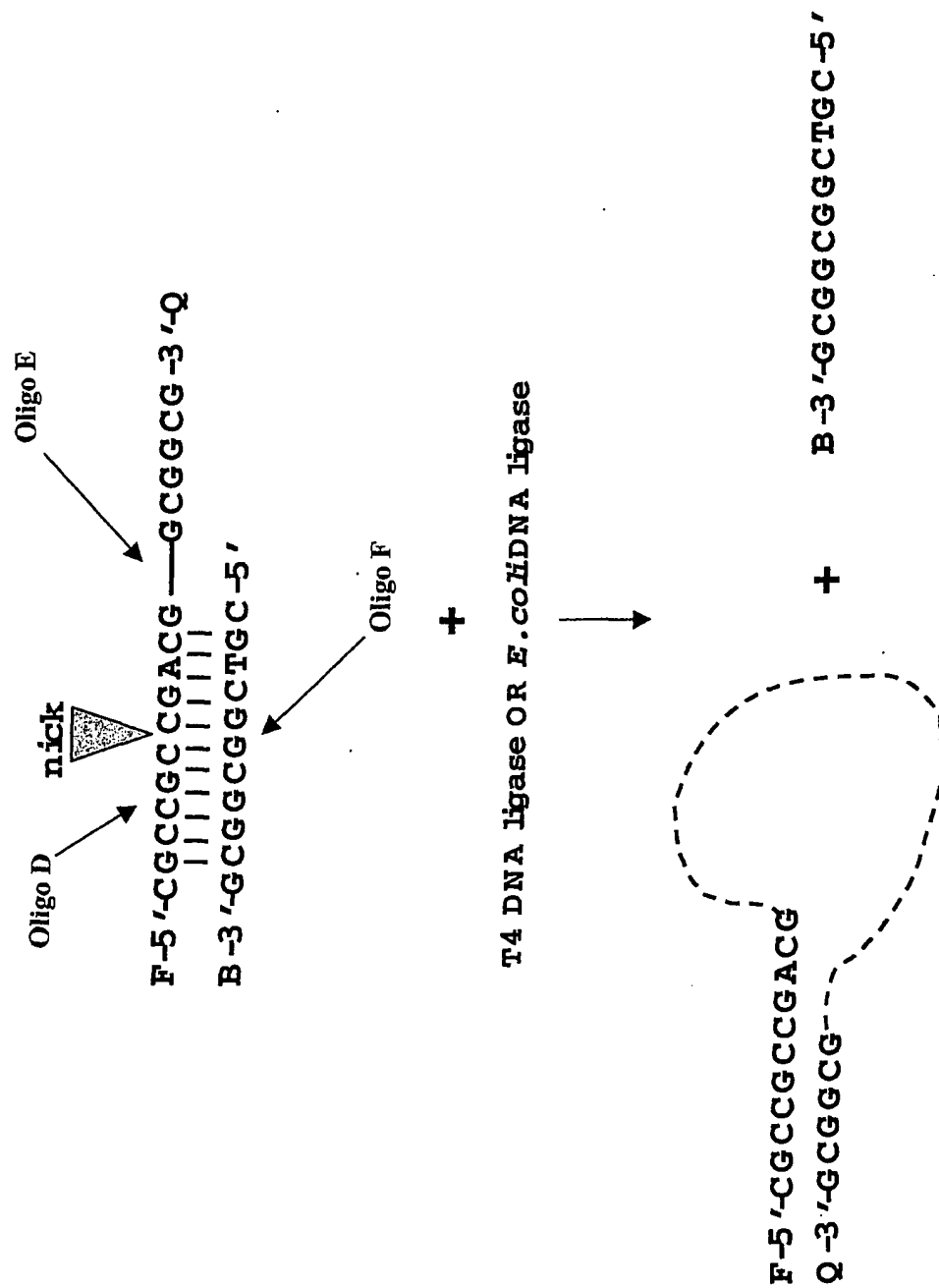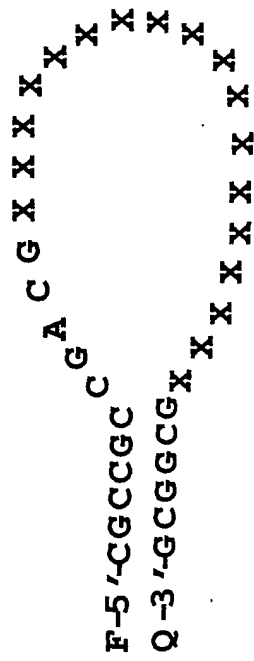
Figure 13

Figure 14

Figure 15:
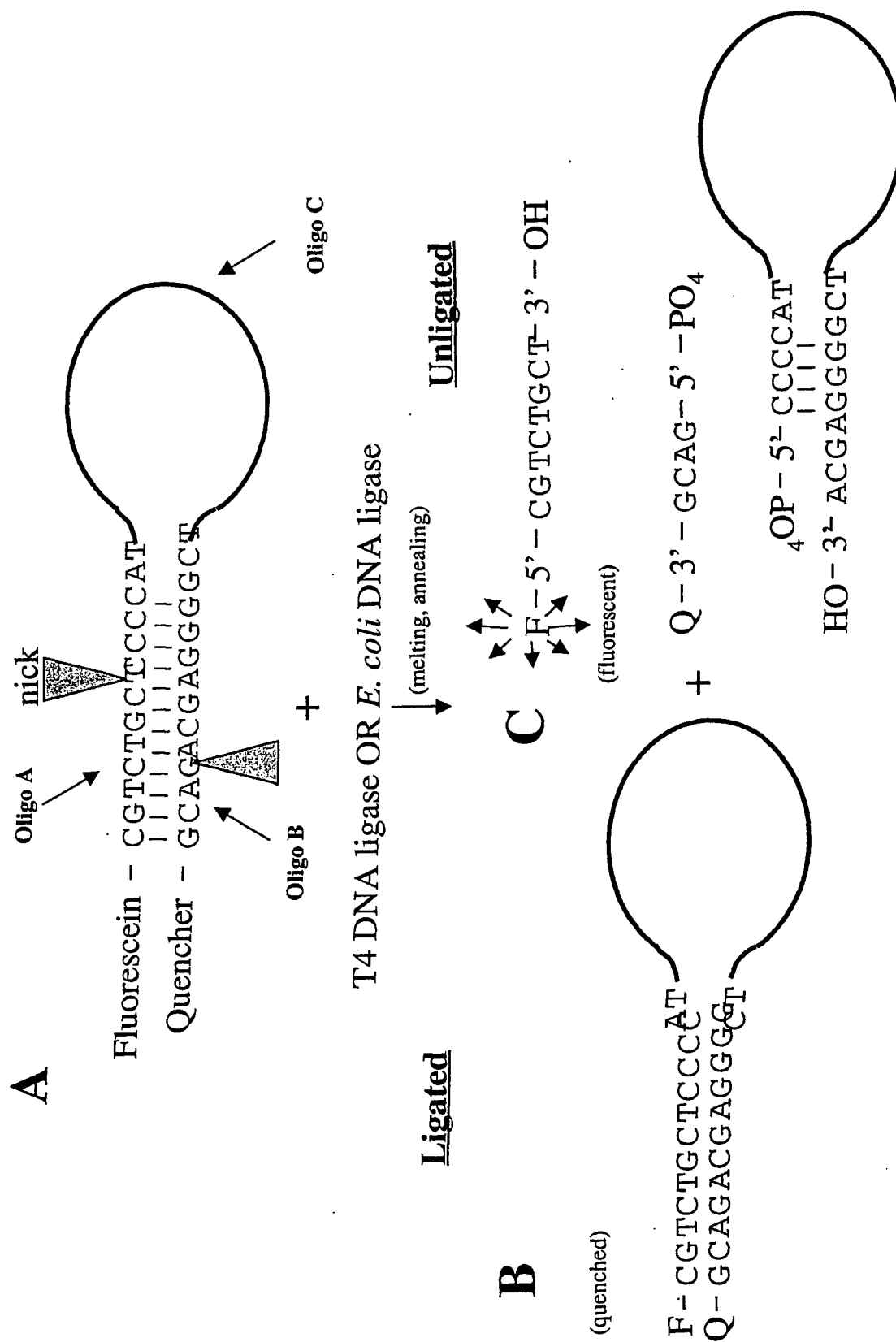
Figure 16:
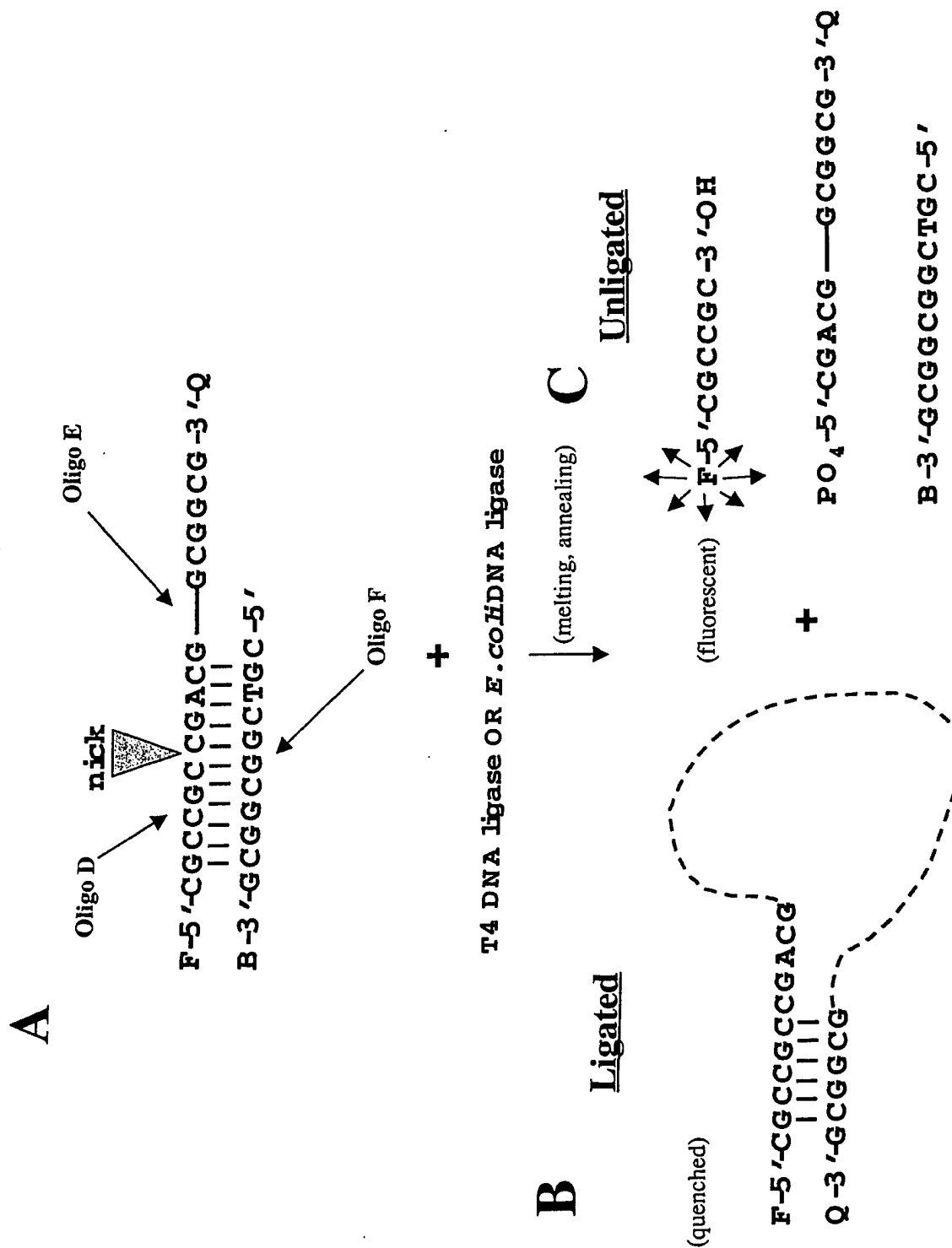
Figure 17

Oligo D

F–5′–CGCCGC

Figure 18

Oligo H →

TAGGCAACTCCAGTAGCGGCG -3'-Q
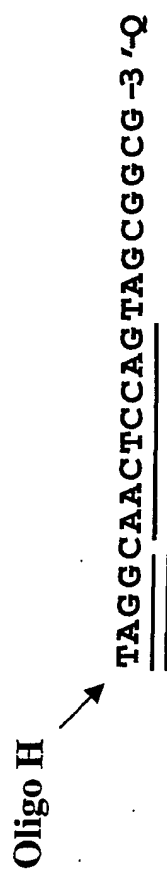
Figure 19

Oligo G

5'-CGACGTAACTCTGAATCGCGC
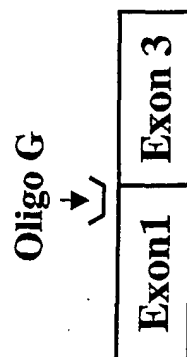
Figure 20

Figure 21
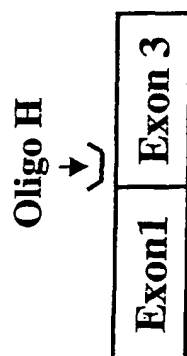
Figure 22

Oligo F

B –3′–GCGGCGGCTGC –5′
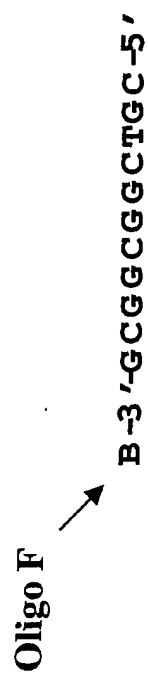
Figure 23

Oligo D

Oligo G

nick

F –5'–CGCCGCCGACGTAACTCTGAATCGCGC
      | | | | | | | | | | |
B –3'–GCGGCGGGCTGC –5'

Oligo F

Oligo I → B –3'–AGCGGCGATCCG –5'

Figure 24

Figure 25

Oligo G

Oligo H

nick

CGACGTAACTCTGAATCGCGCTAGGCAACTCCAGTAGCGGGCG -3'-Q
                    |||||||||||
                B -3'-AGCGCGATCCG -5'

Oligo I

Figure 26

Oligo D   nick   Oligo G   nick   Oligo H

Oligo F   Oligo I

F-5'-CGCCGCCGACGTAACTCTGAATCGGCTAGGCAACTCCAGTAGCGGGCG -3'-Q

B-3'-GCGGCGGCTGC-5'   B-3'-AGCGGCGATCCG-5'
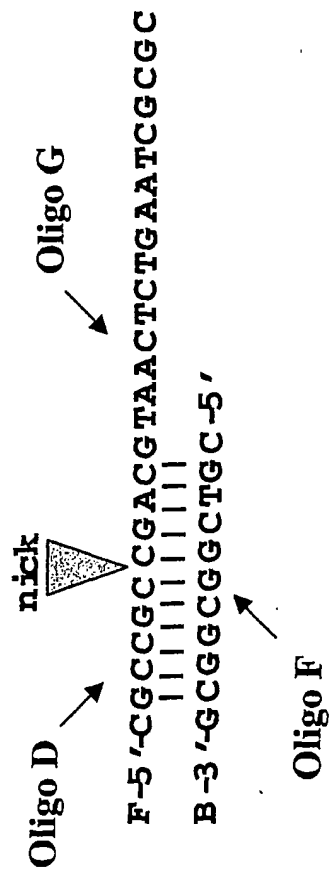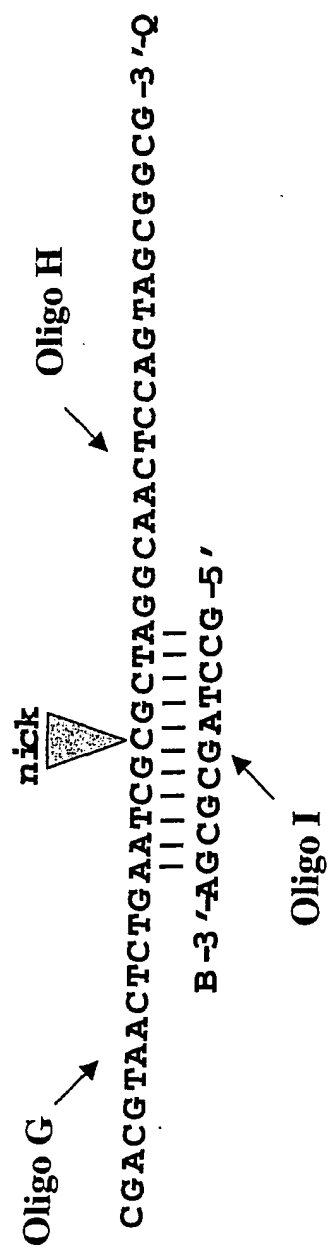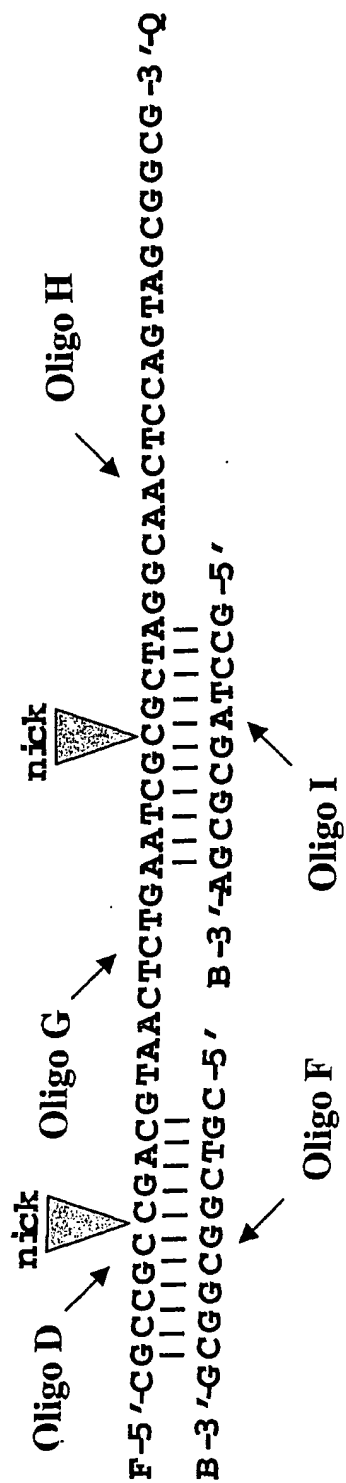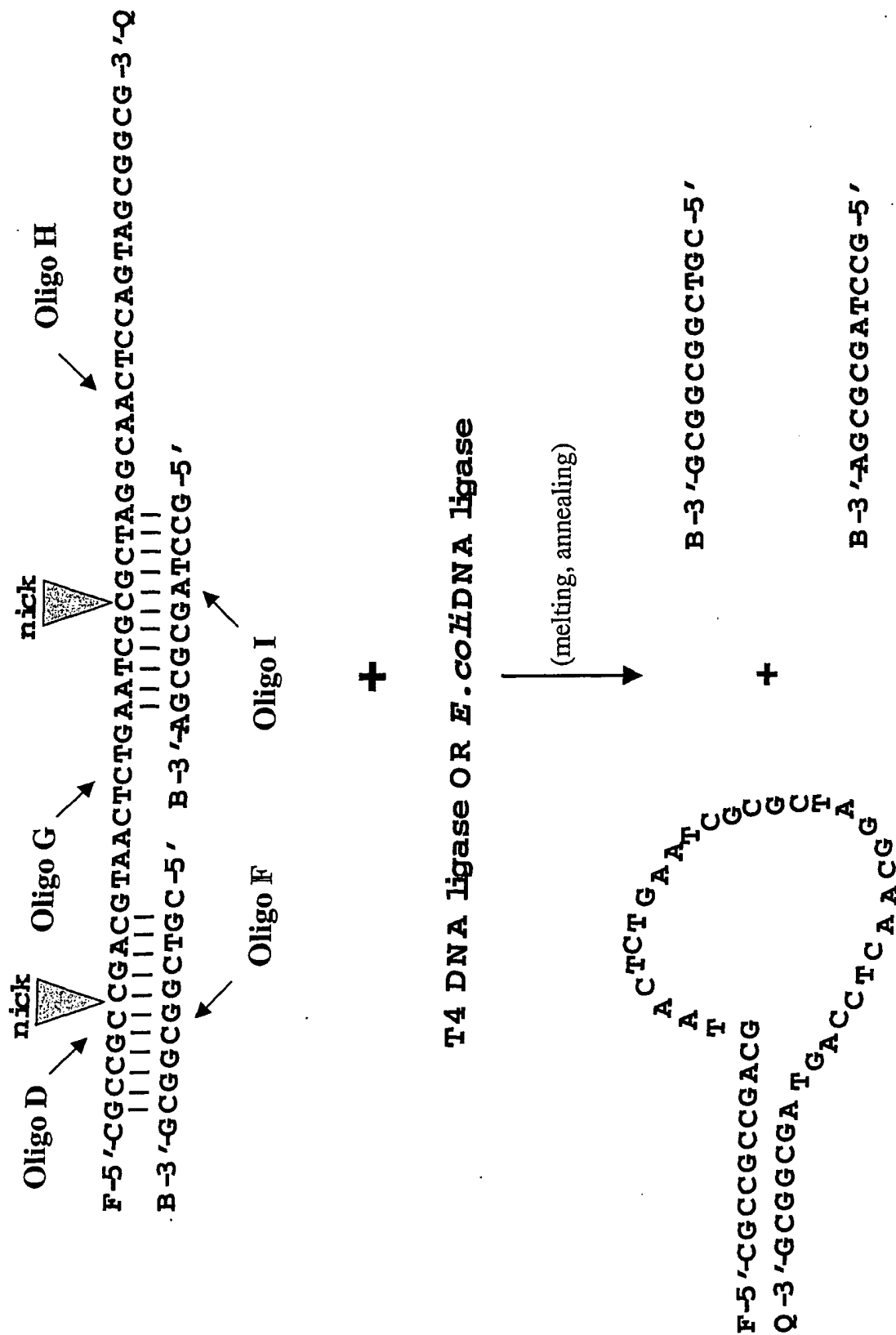
Figure 27

Figure 28

Figure 29
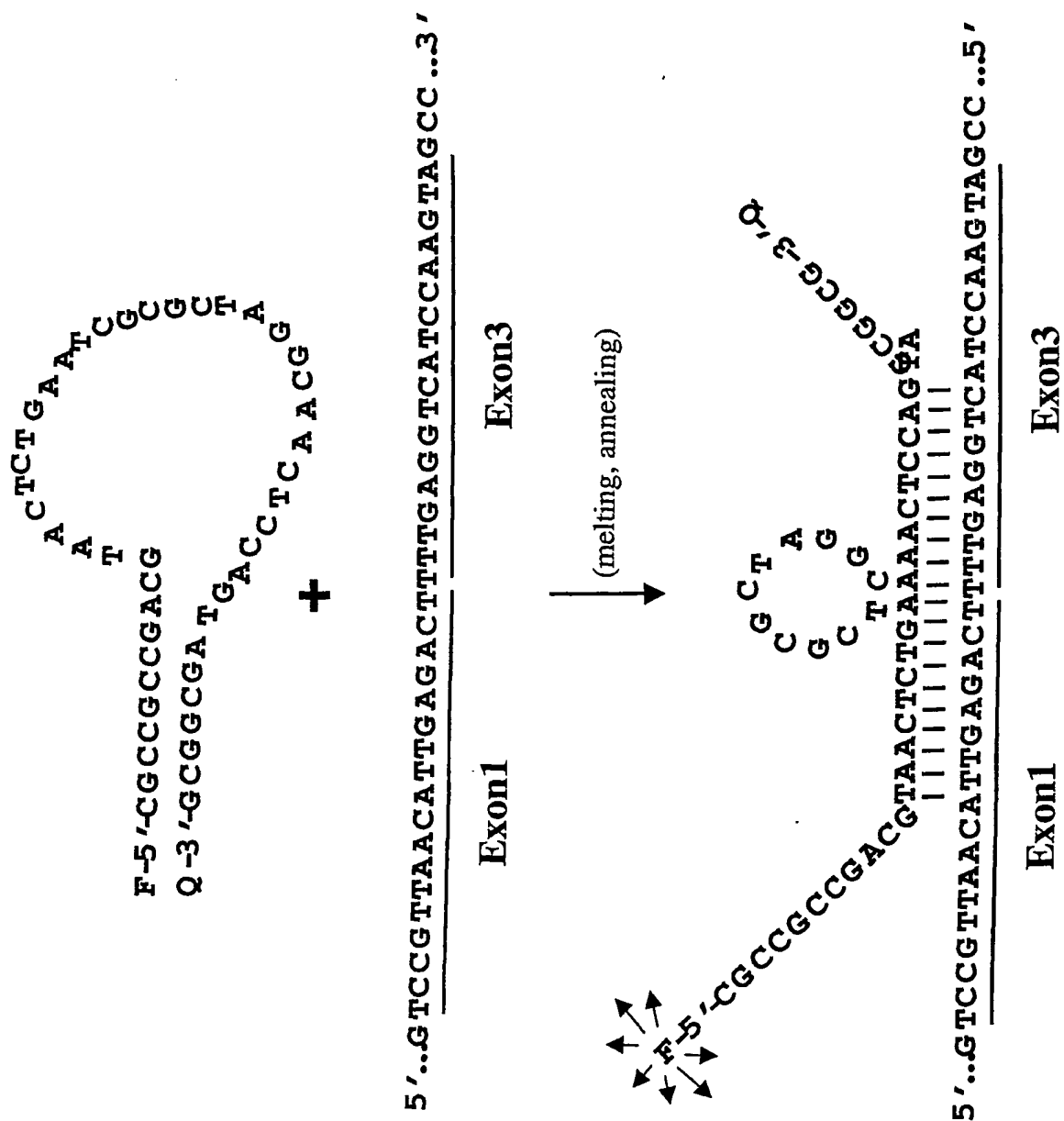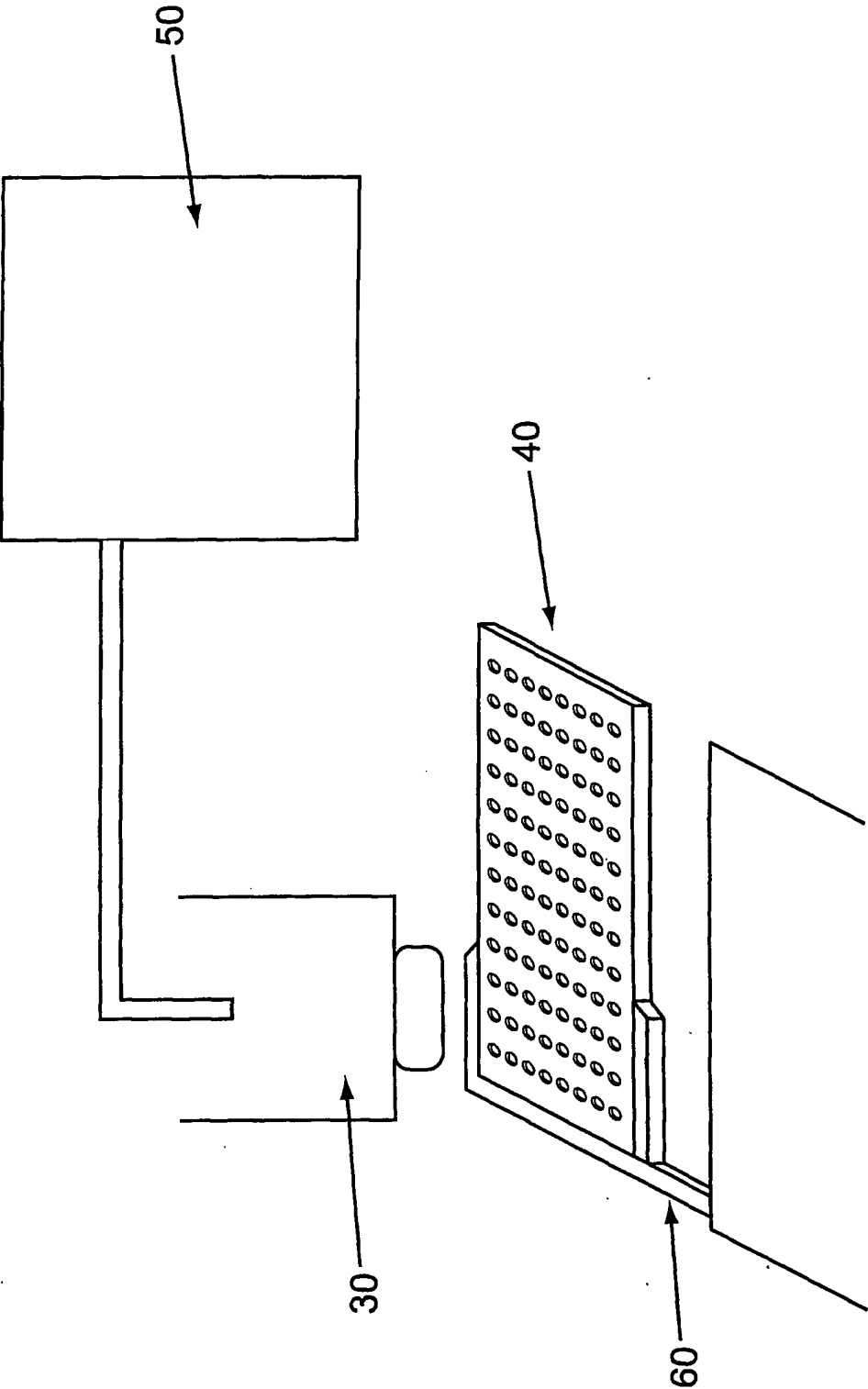
Fig. 30

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34; C07H 21/04
US CL :435/6, 91.1; 536/23.1, 25.3, 25.32
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 536/23.1, 25.3, 25.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, DERWENT WPI, MEDLINE, BIOSIS
search terms: molecular beacon, ligate, ligation, quench, fluorescent, hybridize

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,925,517 A (TYAGI et al.) 20 July 1999, Fig. 4 and col. 27, lines 1-39.	1-21 ------ 22-47

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 JUNE 2001	31 AUG 2001

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